

Differentiation of bone marrow stem cells into functional pancreatic insulin-producing cells

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Abbreviations

β -cells	Beta cells
BM	Bone marrow
BMSC	Bone marrow stem cells
BSA	Bovine serum albumin
bp	Base pair
bHLH	basic helix-loop-helix
cDNA	Complementary Deoxyribonucleic Acid
Cy3	Cyanine-3-Tyramide
DAPI	4', 6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dl	deciliter
ECM	extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ESC	Embryonic stem cells
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
g	Gram
GLP 1	glucagon-like peptide 1
GLUT –2	glucose transporter 2
HBSS	Hank`s balanced salt solution
hr	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HNF	hepatocytes nuclear factor

HSC	Hematopoietic stem cells
IAPP	Islet amyloid polypeptide
IGF	insulin-like growth factor
Isl-1	Islet-1
kDa	Kilo Dalton
l	liter
M	Molar
MODY	maturity-onset diabetes of young
mRNA	messenger Ribonucleic Acid
Min	Minute
MSC	Mesenchymal stem cells
μ	Micron
N	Normal
NGF	nerve growth factor
NGN	neurogenin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDX1/IPF1	pancreatic duodenal homeobox 1/insulin promoter factor 1
PFA	Paraformaldehyde
PP	pancreatic polypeptide
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	rounds per minute
RT	Room temperature/reverse transcriptase
s	Seconds
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	Transmission electron microscopy
TF	Transcription factor
TSA	Trichostatin A

Summary

Diabetes mellitus (DM) is a common metabolic disorder affecting millions of people worldwide and is characterized by abnormally high levels of glucose in blood. Type 1 diabetes is caused by the destruction of pancreatic β -cells by T cells of the immune system. The common therapy of diabetes is daily injections of insulin. However, this therapy does not overcome the serious long-term complications that, result in overall shortened life expectancy of diabetic patients. Therefore, great efforts have been undertaken to develop novel strategies. A promising alternative to present treatments would be the generation and implantation of pancreatic islets from adult stem cells.

Conditions were investigated that allow BM stem cells (BMSC) to differentiate into insulin-producing cells. A novel *in vitro* differentiation method was developed by using the histone deacetylase inhibitor (HDACi), Trichostatin A (TSA). BMSC, cultured in the presence of TSA. BMSCs differentiated into islet-like clusters under these culture conditions. These clusters were similar to the cells of the islets of the pancreas. The cells in the clusters showed endoderm specific gene expression typical for pancreatic β -cell development and function, such as insulin (I and II), glucagon, somatostatin, GLUT-2, pancreatic duodenal homeobox-1 (PDX-1), and Pax 4. To show that cells of the islet-like clusters synthesized pancreatic hormones, the co-localization of insulin and C-Peptide

was analyzed by immunocytochemistry. Enzyme-linked immunosorbent assay (ELISA) analysis demonstrated that insulin secretion was regulated by glucose concentration. Western blot analysis showed the presence of intracellular stored insulin. Electron microscopy of the islet-like cells revealed that they possessed an ultrastructure similar to that of pancreatic β -cells, which contain insulin granules within secretory vesicles. These findings suggest that histone-deacetylating agents help to promote differentiation of BMSC into functional insulin-producing β -cells. In addition, gene expression analysis confirmed the presence of new signaling pathways that are important in endocrine pancreas development. A subset of known and novel genes was temporally regulated, including genes that spatially define developing endocrine cells from BMSC. The development of new protocols to stimulate the differentiation of BMSC into pancreatic β -like cells may contribute to future cell therapeutic treatments of type 1 diabetes.

1. Introduction

A major source of energy for all cells is glucose, and all major food types are first broken-down to glucose prior to being used. Glucose is released from the gut into the blood stream, is taken up by cells in all tissues of the body, and is used as a fuel to generate energy that allows us to undertake all our major activities such as walking, running, and reading. Any excess sugar that is not used up immediately is stored in the cells as a backup source of energy. In healthy individuals, the level of circulating blood sugar is strictly regulated. This state of equilibrium, normoglycemia (normal glucose level), is tightly controlled by the hormone insulin, which is secreted in specific cells in the pancreas, the β -cells. This balance in glucose level is achieved through the regulation of the amount of insulin that is secreted to down-regulate increased blood sugar levels after consumption of sugar containing nutrients.

1.1 Regulation of blood glucose

Blood glucose levels are not always constant; they rise and fall depending upon the body's needs and are regulated by hormones. This results in glucose levels normally ranging from 70 to 110 mg/dl. The blood glucose level can rise for three reasons: diet, breakdown of glycogen, or hepatic synthesis of glucose. Eating produces a rise in blood glucose, the extent of which depends on a number of factors such as the amount and the type of carbohydrate eaten (i.e., the glycemic index), the rate of digestion, and the rate of

absorption. Because glucose is a polar molecule, its absorption across the hydrophobic gut wall requires specialized glucose transporters (GLUTS).

The liver releases glucose from the breakdown of glycogen and also synthesizes glucose from intermediates of carbohydrate, protein, and fat metabolism. The liver is a major regulator of glucose and can buffer blood glucose levels. It receives glucose-rich blood directly from the digestive tract via the portal vein. The portal vein drains almost all of the blood coming from the digestive tract and empties directly into the liver. The liver can quickly remove large amounts of glucose from the circulation so that even after a meal, blood glucose levels rarely rise above 110 mg/dl.

The rise in blood glucose following a meal is detected by the pancreatic beta (β) cells, which respond by releasing insulin. Insulin increases the uptake and use of glucose by tissues such as skeletal muscle and fat cells. This rise in glucose also inhibits the release of glucagon, inhibiting the production of glucose from other sources, e.g., by glycogen breakdown.

Once inside the cell, some of the glucose is used immediately via glycolysis. During the well-fed state, the high levels of insulin and low levels of glucagon stimulate glycolysis, which releases energy and produces carbohydrate intermediates that can be used in other metabolic pathways. Brain consumes more than 80% glucose. The liver and muscle take up any glucose that is not used immediately, where it can be converted into glycogen. Insulin stimulates glycogenesis in the liver by inducing hepatic glycogen synthetase (the enzyme that catalyzes glycogen synthesis in the liver). Insulin prevents the breakdown of stored glycogen (glycogenolysis) and the synthesis of new glucose (gluconeogenesis). Insulin also encourages glycogen formation in muscle, but by a different method. Here, it

increases the number of glucose transporters (GLUT4) on the cell surface. This leads to a rapid uptake of glucose that is converted into muscle glycogen.

When glycogen stores are fully replenished, excess glucose is converted into fat in a process called lipogenesis. Glucose is converted into fatty acids that are further processed to triglycerides for storage. Insulin promotes lipogenesis by increasing the number of glucose transporters (GLUT4) expressed on the surface of the fat cell, causing a rapid uptake of glucose. In addition to promoting fat synthesis, insulin also inhibits fat breakdown by inhibiting hormone-sensitive lipase. As a result, levels of fatty acids in the blood stream decrease. Insulin also has an anabolic effect on protein metabolism. It stimulates both the entry of amino acids into cells and protein production from amino acids.

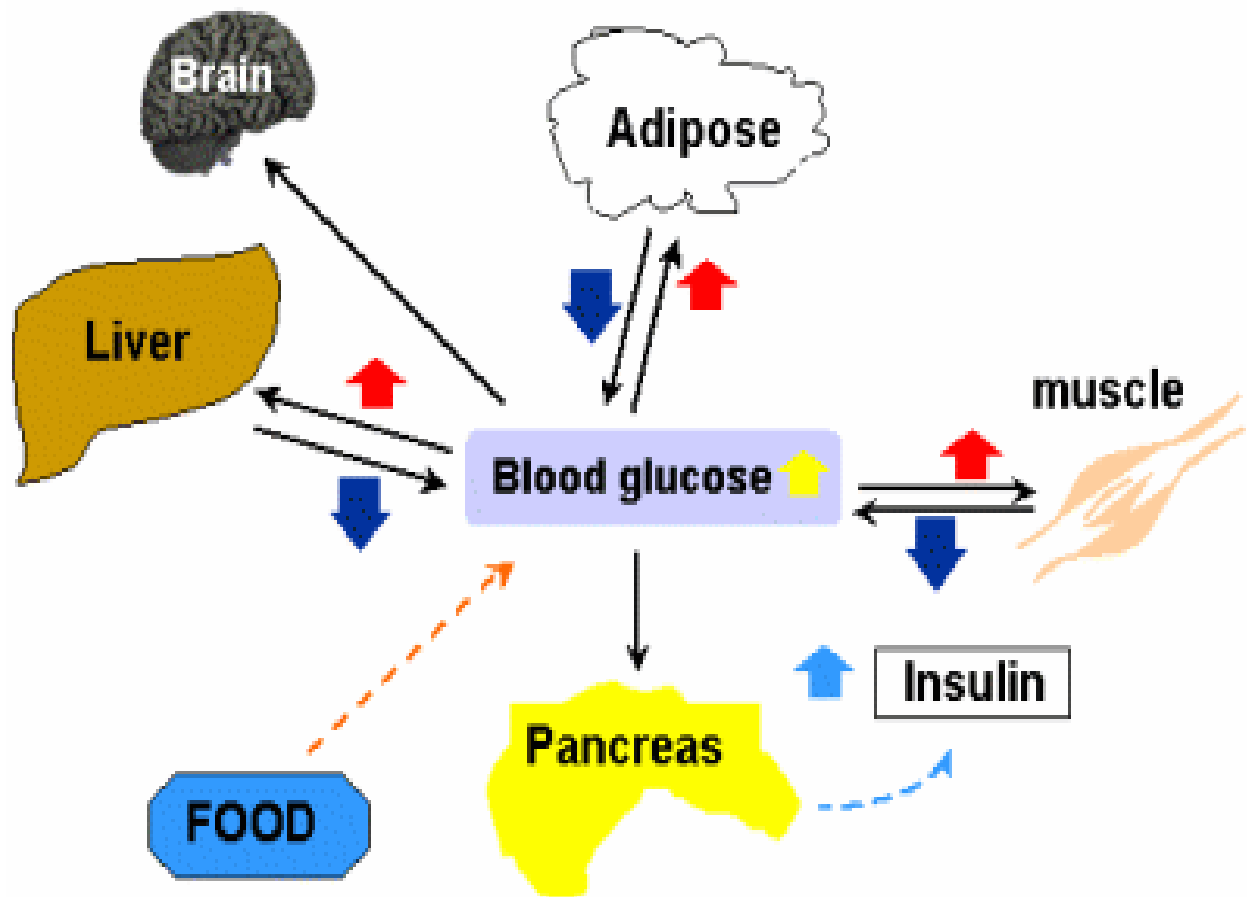


Figure 1.1 **Mechanisms of glucose balance**

Under basal conditions, 80% of circulating glucose is taken up by the brain. When food is ingested, there is a parallel rise in blood glucose level (yellow arrow). This increase in blood glucose is sensed by the β -cells in the pancreas, and as a result, insulin is secreted (pale blue arrows). Insulin circulates through the body and signals to the major insulin sensitive organs (muscle, liver, and fat) to increase their glucose intake (red arrows). Insulin simultaneously leads to a reduction of glucose production from the liver and other organs (blue arrows). In this way, the hormone insulin counteracts the rise of glucose in the blood, thereby returning the system to its equilibrium. (*This figure is adopted from Sanger Institute*).

1.1.1 Role of Glucagon

A drop in blood sugar levels inhibits insulin secretion and stimulates glucagon release. Glucagon opposes many actions of insulin. Most importantly, glucagon raises blood sugar levels by stimulating the mobilization of glycogen stores in the liver, providing a rapid burst of glucose. During continued fasting, glycogen stores are depleted under such conditions; glucagon stimulates glucose production by stimulating the hepatic uptake of amino acids, the carbon skeletons of which are used to synthesize glucose.

1.2 Anatomy and functions of pancreas

1.2.1 Anatomy

The human pancreas is an elongated tapered organ lying behind the stomach and across the back of the abdomen. The pancreas is made up of two types of tissues: exocrine and endocrine tissue. The cells of the exocrine tissue secrete digestive enzymes into the digestive tract. These enzymes break down all categories of digestible foods. The endocrine pancreas produces hormones that affect carbohydrate metabolism. The endocrine tissue, which consists of the islets of Langerhans, secretes these hormones into the bloodstream. The islets of Langerhans contain insulin-producing β -cells (60–80% of the islet cells), glucagon-releasing alpha-cells (10-20%), somatostatin-secreting delta-cells (3-10%), and a few pancreatic polypeptide-containing (PP) cells. Most of the insulin-containing cells occur in cell groups in the islets of Langerhans. The number and size of the pancreatic islets vary among species and seem to be regulated by developmental, nutrient, and hormonal factors. Pancreatic β -cells compose more than 60% of the adult islet volume. Single islet β -cells rapidly and spontaneously reaggregate into spherical particles that can establish a mantle by associating with other islet

endocrine cells. This reaggregation of islet β -cells is thought to be calcium-dependent and stimulated by glucose and cyclic AMP.

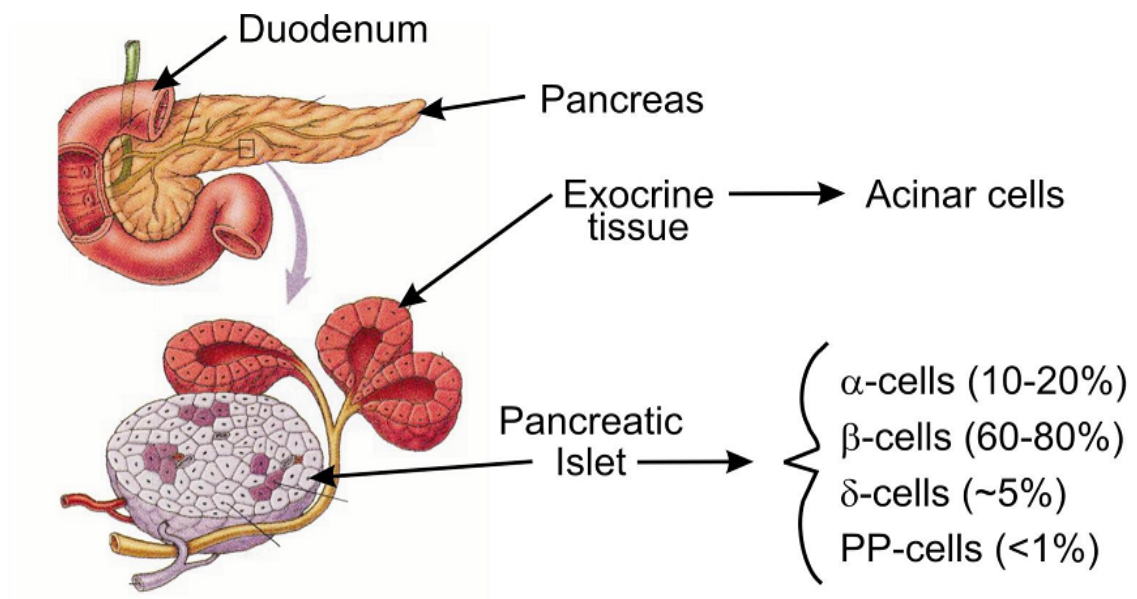


Figure.1.2 Schematic diagram of the anatomy of the pancreas

The pancreas is located in the curve of duodenum. The exocrine pancreas contains acinar cells, whereas the endocrine pancreas contains four different populations of cells: alpha-cells, β -cells, delta-cells, and PP cells. (This figure is adopted from the website <http://www.uchsc.edu/misc/diabetes/Chapter2C.ppt>)

1.2.2 General functions

The pancreas has digestive and hormonal functions. The enzymes secreted by the exocrine tissue in the pancreas help to break down carbohydrates, fats, proteins, and acids in the duodenum. These enzymes travel down the pancreatic duct into the bile duct in an

inactive form. When they enter the duodenum, they are activated. The exocrine tissue also secretes bicarbonate to neutralize stomach acid in the duodenum. The hormones secreted by the endocrine tissue in the pancreas are insulin and glucagon (which regulate the level of glucose in the blood), and somatostatin (which prevents the release of the other two hormones).

1.2.3 Functions of pancreatic β -cells

The main function of pancreatic β -cells is the production and controlled release of insulin. The insulin gene is present as a single copy in all species, with the exception of rat and mouse, where there are two non-allelic insulin genes. The human insulin gene is located near the end of the short arm of chromosome 11 and is flanked on the 5'-side with the gene for tyrosine hydroxylase and on the 3'-side with the gene for IGF-II. The structure of the gene is highly conserved.

Insulin is a small hormone composed of two polypeptide chains (A and B) linked by two disulfide bonds and has a molecular weight of about 6000 daltons. The A chain consists of 21 amino acids and the B chain of 30 amino acids. Insulin is a small protein and is produced as part of a larger protein to ensure it folds properly. In the protein assembly of insulin, the messenger RNA transcript is translated into an inactive protein called preproinsulin (Figure 1.3). Preproinsulin contains an amino terminal signal sequence that is required in order for the precursor hormone to pass through the membrane of the endoplasmic reticulum(ER) for post-translational processing. The post-translational processing clips away those portions not needed for the bioactive hormone. Upon entering the ER, the preproinsulin signal sequence, now useless, is proteolytically removed from proinsulin. Once the post-translational formation of three vital disulfide

bonds occurs, specific peptidases cleave proinsulin. The final product of the biosynthesis is mature and active insulin. Finally, insulin is packaged and stored in secretory granules, which accumulate in the cytoplasm, until release is triggered.

The secretory vesicles contain up to 80% of insulin related proteins. These are principally insulin and C-Peptide, both being present in equimolar amounts, and a much smaller amount of proinsulin and its intermediate conversion products. Insulin molecules are stored in crystalline form, occupying the electron-dense portion of the secretory granules. An adult pancreatic β -cell has a store of approximately 50 pg insulin per cell. Insulin molecules in storage granules are secreted by granule membrane fusion with the plasma membrane of the cell. Insulin molecules have a tendency to form dimers in solution because of hydrogen-bonding between the C-termini of the B chains. Additionally, in the presence of zinc ions, insulin dimers associate into hexamers.

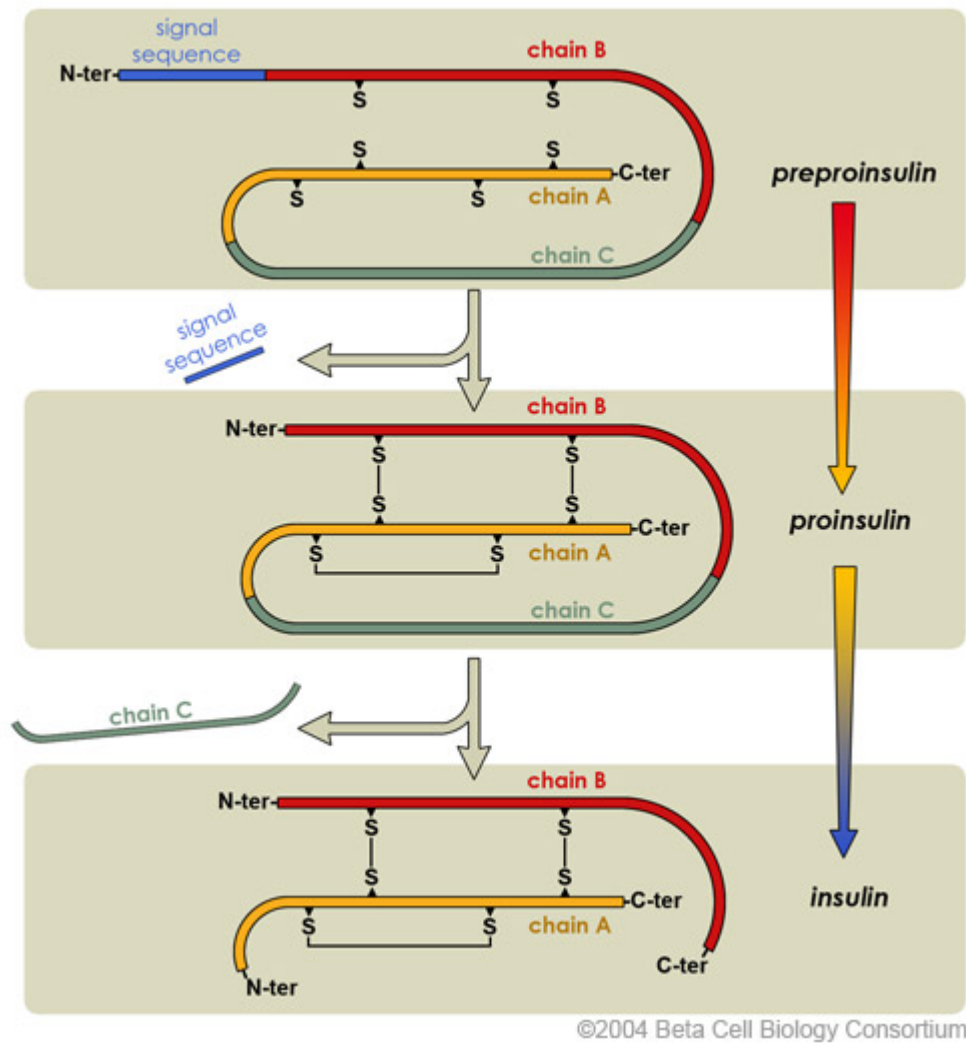


Figure 1.3 **Schematic representation of insulin synthesis**

Insulin production involved intermediate steps. Initially, preproinsulin is the inactive form that is secreted into the endoplasmic reticulum. Post-translational processing clips the N-terminal signal sequence and forms the disulfide bridges. Lastly, the polypeptide is clipped at two positions to release the intervening chain C. This and active insulin are finally packaged into secretory granules for storage. (*This picture is adopted from Beta cell biology consortium*)

1.2.4 Insulin secretion

Increasing levels of glucose (above 10 mM) inside the pancreatic β -cells trigger the release of insulin. However, insulin release is also induced by other factors, such as high amino acid and fatty acid levels in the blood, hormones released from the stomach and intestine, and neurotransmitters (Lang, 1999).

Glucose is transported into the β -cell by type 2 glucose transporters (GLUT2). Once inside, the first step in glucose metabolism is the phosphorylation of glucose to produce glucose-6-phosphate. This step is catalyzed by glucokinase; this is the rate-limiting step in glycolysis, and it effectively traps glucose inside the cell. As glucose metabolism proceeds, ATP is produced in the mitochondria. The increase in the ATP:ADP ratio closes ATP-gated potassium channels in the β -cell membrane. Positively charged potassium ions (K^+) are now prevented from leaving the β -cell. The rise in the positive charge inside the β -cell causes depolarization. Voltage-gated calcium channels open, allowing calcium ions (Ca^{2+}) to flood into the cell. The increase in the intracellular calcium concentration triggers the secretion of insulin via exocytosis (Figure 1.4).

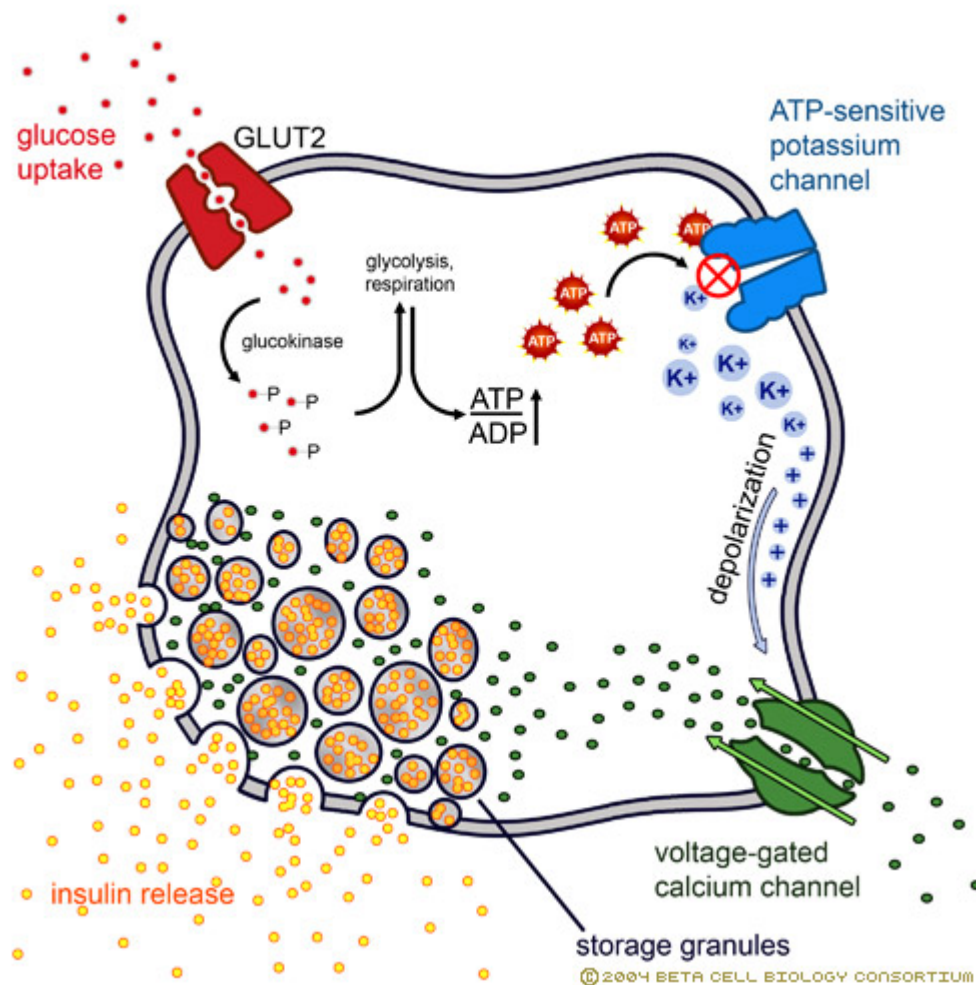


Figure 1.4 **Schematic presentation of mechanism of insulin secretion**

Insulin secretion in beta cells is triggered by rising blood glucose levels. Starting with the uptake of glucose by the GLUT2 transporter, the glycolytic phosphorylation of glucose causes a rise in the ATP:ADP ratio. The rise inactivates the potassium channel that depolarizes the membrane, causing the calcium channel to open up allowing calcium ions to flow inward. The ensuing rise in levels of calcium leads to the exocytotic release of insulin from their storage granule. (*This picture is adopted from Beta cell biology consortium.*)

There are two phases of insulin release in response to a rise in glucose. The first is an immediate release of insulin. This is attributable to the release of preformed insulin, which is stored in secretory granules. Once depleted, a second phase of insulin release is initiated. This latter release is prolonged since insulin has to be synthesized, processed, and secreted for the duration of the increase of blood glucose. Furthermore, beta cells also have to regenerate the stores of insulin initially depleted in the fast response phase. As important as insulin is to preventing too high of a blood glucose level, it is just as important that there not be too much insulin and hypoglycemia. As one step in monitoring insulin levels, the enzyme insulinase (found in the liver and kidneys) breaks down blood-circulating insulin resulting in a half-life of about six minutes for the hormone. This degradative process ensures that levels of circulating insulin are modulated and that blood glucose levels do not get dangerously low.

1.3 Pancreas organogenesis

After gastrulation, mammalian endoderm, which is a sheet of undifferentiated cells, eventually forms the epithelial components of the gastrointestinal tract (esophagus, stomach, and intestine) and the glands derived from it (thyroid, thymus, pancreas, and liver) (Well et al., 1999). Pancreas arises from the endoderm as a dorsal and a ventral bud that fuse together to form a single organ (Slack, 1995).

In mice, the pancreas begins to develop at embryonic day E9.5 from evaginations of the foregut endoderm at the level of the anterior intestinal portal (AIP), forming two primordia (dorsal and ventral) capped by mesenchymal cells (Pictet et al., 1972, Wells and Melton, 1999, Wessels and Cohen, 1967), which subsequently form a single gland at

around E13.5. This period of pancreatic organogenesis is called primary transition, referring to the changing of the pre-pancreatic endoderm (Edlund, 1999, 2000). Proliferation of the pancreatic epithelium results in the formation of branching cords from which clusters of endocrine and exocrine cells progressively differentiate (Herrera et al., 1991, Slack, 1995). This differentiation wave occurs during the secondary transition, between E13.5 and E15.5 (Pictet and Rutter et al., 1972).

1.4 Genes and transcription factors involved in the development of pancreas

The genes and transcription factors involved in the pancreas development were listed in Table 1.

Table 1. Major pancreatic transcription factors; their expression and functions

Transcription factor	Onset and site of pancreatic expression	Mouse & human mutations	References
Homeodomain factors			
Pdx1	e8.5, early pancreatic epithelium, beta and delta cells of mature pancreas	heterozygous human mutation: MODY4 Pancreatic agenesis,	Guz et al., 1995; Jonsson et al., 1994; Stoffers et al.1997a,b Ahlgren et al. 1998;
Pbx1	e10.5, ubiquitous	Hypoplastic pancreas	Kim et al., 2002
HNF6	e9.5, throughout the pancreatic buds	Impaired endocrine differentiation	Jacquemin et al.,2000
Pax4	e9.5, in developing endocrine cells, not found in mature pancreas	Deficiency of beta & delta cells, increase in alpha cells	Dohrmann et al.,2000; Sosa-Pineda et al., 1997
Pax6	e9, in endocrine cells	No alpha cells, decrease in other endocrine cell types	St-Onge et al., 1997
Nkx2.2	e9.5, throughout pancreatic bud, later in endocrine cells	Lack of beta cells, decrease in alpha and PP cells	Sussel et. al, 1998
Nkx6.1	e9.5, in beta cells	Decrease in beta cells	Sander et al., 2000
Isl1	e9, in all islet cells	No differentiated islet cells	Ahlgren et al., 1997

HNF1a	e10.5, dorsal pancreas, mature beta cells	Heterozygous human mutation and homozygous mouse mutation: MODY3	Nammo et al., 2002; Pontoglio et al., 1998
HNF4a	e5.5, in visceral endoderm, e9.5 in pancreatic primordium	Early embryonic lethal Heterozygous human mutation: MODY1	Ferrer, 2002; Duncan et al., 1994

Basic helix-loop-helix factors

Ngn3	e9.5, endocrine precursors	No endocrine cells	Gradwohl et al., 2000; Gu et al., 2002
NeuroD	e9.5, endocrine precursors and adult islet cells	Impaired islet development	Jensen et al., 2000a; Naya et al., 1997
Ptf1-p48	e10, exocrine, endocrine and ductal progenitors	No exocrine cells	Kawaguchi et al., 2002; Krapp et al., 1996; Krapp et al., 1998

Forkhead / winged helix factors

Foxa2 / HNF3b	e5.5-6.5, early foregut endoderm, adult acinar and islet tissue	Lack of foregut formation	Lee et al., 2002; Sund et al., 2001
Foxa1 / HNF3a	e7.5, notochord, later in all islet cell types	Reduced proglucagon expression	Kaestner et al., 1999

1.5 Pancreatic diseases

A number of diseases relate to the pancreas. The most frequently occurring diseases are pancreatic carcinoma, pancreatitis (acute or chronic), pancreatic enzyme insufficiency and diabetes mellitus.

Some 30,000 Americans are diagnosed with pancreatic cancer each year, and nearly all will succumb to the disease.

Pancreatitis is a rapid and severe inflammation of pancreas; it can be acute or chronic. Binge alcohol drinking is a common cause of acute pancreatitis. Gallstones lodged in the main bile duct next to the pancreas also cause this condition. Accidents, such as the upper abdomen hitting the steering wheel during a car accident, can cause pancreatitis. Certain drugs, such as diuretics, can produce the disorder, as can extremely high blood fat levels (triglycerides). Heredity seems to play a role since, in some families; the condition develops in several members of the family. In pancreatitis, the digestive enzymes of the pancreas break out into the tissues of the organ rather than staying within the tubes (ducts). Severe damage to the pancreas then results.

Pancreas enzyme insufficiency occurs when there is deficiency in the digestive enzymes that are necessary to break down protein, fat, and carbohydrates in foods that are ingested. The nutrients are not broken down, resulting in malnutrition and weight loss. This condition is also called malabsorption because the intestine is unable to absorb these vital nutrients.

1.6 Diabetes mellitus

According to the World Health Organization in 2006, at least 171 million people worldwide suffer from diabetes. Diabetes mellitus is characterized by abnormally high levels of glucose in blood. This may be because of the inadequate secretion or action of insulin, an inadequate response by the body's cells to insulin, or a combination of all these factors. The most common forms of diabetes are type 1 and type 2 diabetes. Gestational diabetes occurs at the time of pregnancy, and other forms of diabetes are rare and are caused by a single gene mutation.

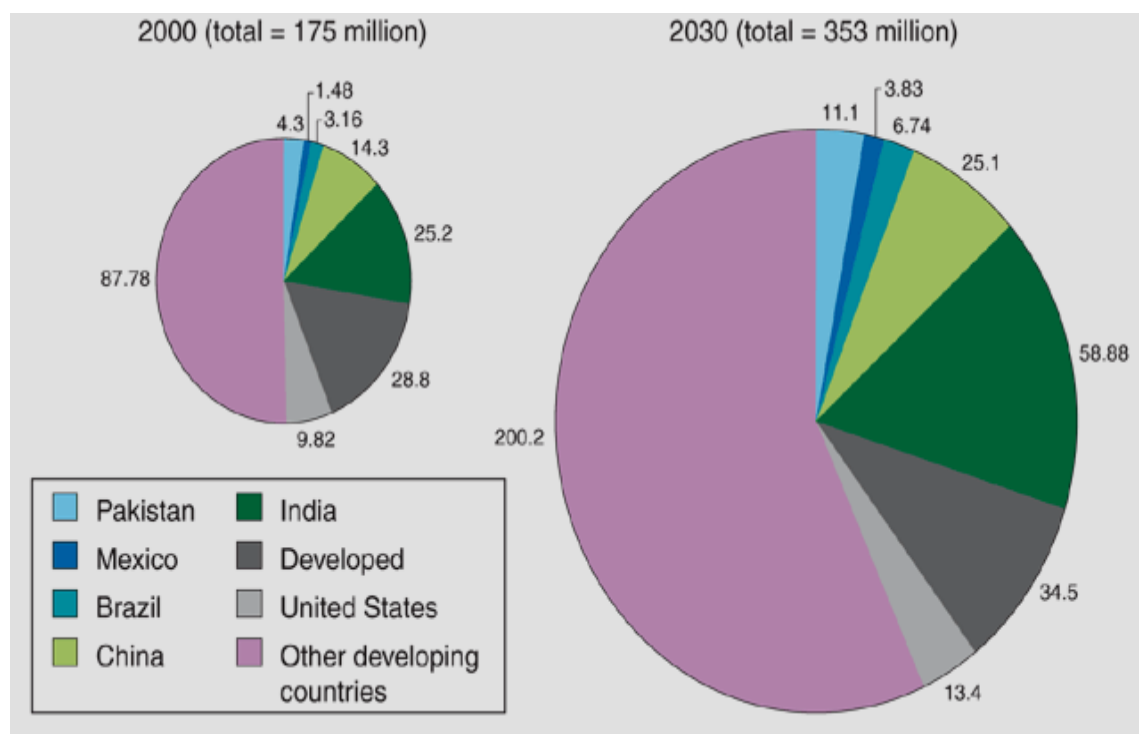


Figure 1.5 **Epidemiology of diabetes**

Number of persons with diabetes in the year 2000 and estimated for the year 2030. (*This figure is adopted from Yach et al., 2006.*)

1.6.1 Type 1 diabetes

Type 1 diabetes affects 700,000 people in the United States and is the most common chronic metabolic disorder to affect children. Type 1 diabetes (10% of cases) is caused by autoimmune destruction of β -cells of the islets of Langerhans resulting in absolute insulin deficiency. The gene thought to be responsible for this reaction is located within a region of the major histocompatibility complex (MHC) HLA class 2 gene, called IDDM1. The role of this gene in the immune response is the presentation of foreign molecules to T cells; however, the detailed action of the gene is still under investigation. The onset is usually acute, the disease developing over a period of a few days to weeks. Over 95% of persons with type 1 diabetes mellitus develop the disease before the age of 25, with an equal incidence in both sexes.

1.6.2 Type 2 diabetes

An important factor in type 2 diabetes is the body's resistance to insulin, and another factor is the falling production of insulin by the β -cells of pancreas. Thus, type 2 diabetes may be a combination of the deficient secretion and deficient action of insulin. Defective β -cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycemia. This is the most common form of diabetes mellitus, and the etiology is multifactorial. It is highly associated with a family history of diabetes, older age, obesity, and lack of exercise. Type 2 diabetes accounts for 90-95% of all cases of diabetes. The prevalence of type 2 diabetes is increasing dramatically across the globe and in some areas has reached epidemic proportions. This increase in prevalence is primarily being driven by environmental factors, through "modern age" dietary and exercise habits.

1.7 Pathology of diabetes

People with diabetes are vulnerable to a variety of complications over time. Control of blood sugar is the best way to minimize the risk of complications. However, even the best control may not be able to eliminate all complications, and the risk of side effects increase with time.

1.7.1 Short-term acute complications

In the absence of either the secretion or action of insulin, the blood glucose concentration rises quickly (hyperglycemia) after glucose or carbohydrate intake. As a result, the amount of glucose that is filtered into the kidney tubules increases. The capacity for the kidney to reabsorb glucose from the urine is limited. If the amount of glucose that enters the tubules is too high (i.e., if it exceeds the glucose threshold), glucose appears in the urine. This condition is termed glucosuria. Because of osmotic effects, glucose in the urine draws with it considerable amounts of water, which will be excreted along with the glucose. As a result, urine volume and frequency increases (polyuria) and the diabetic individual are frequently dehydrated and nearly always thirsty (polydipsia).

In type 1 diabetics, the unopposed actions of glucagon result in increased ketone formation by the liver. These ketones are acidic, and their build up considerably lowers blood pH and disturbs the acid/base balance of the body. This condition is termed ketoacidosis and is the most frequent cause of death amongst diabetics under the age of 30. When ketones reach high enough concentrations in the blood, they are excreted out with the urine. Ketones carry cations, such as sodium (Na^+) and potassium (K^+), with them into the urine leading to electrolyte imbalances in the body. Consequences of these

complications may include abdominal pain, vomiting, sweet-smelling breath, and severe dehydration. Severe cases can lead to diabetic coma and death.

1.7.2 Long-term chronic complications

Several secondary complications usually accompany long-standing diabetes mellitus. These often involve gradual changes that develop over a period of years and may shorten the life expectancy of diabetic individuals. The most common involve the damage of blood vessels. In diabetes, the resultant problems are grouped under “microvascular disease” (caused by damage to small blood vessels) and “macrovascular disease” (caused by damage to the arteries).

1.7.3 Microvascular disease

The damage to small blood vessels leads to microangiopathy, which causes organ-related problems. Lesions in the small blood vessels and capillaries supplying the retina of the eye lead to diabetic retinopathy. Every year thousands of diabetics become blind as a result. Abnormal and decreased sensation, usually progressive, starting at the feet but potentially in other nerves called as diabetic neuropathy. Combined with damage in blood vessels, this can lead to diabetic foot. Other forms of diabetic neuropathy may present as mononeuritis or autonomic neuropathy. Damage to the kidney, which can lead to chronic renal failure, eventually requiring dialysis also known as diabetic nephropathy. Diabetes is the most common cause of adult kidney failure worldwide.

1.7.4 Macrovascular disease

Macrovascular disease leads to cardiovascular disease, mainly by accelerating atherosclerosis. Coronary artery disease can lead to myocardial infarction (“heart attack”)

or angina. Diabetes can also cause stroke (mainly ischemic type) and diabetic myonecrosis.

Long-term microvascular and neurologic complications cause major morbidity and mortality in patients with insulin-dependent diabetes mellitus. Careful maintenance of blood concentrations close to the normal range can decrease the frequency and severity of these complications.

1.8 Treatment of diabetes mellitus

1.8.1 Treatment for type 1 diabetes Type 1 diabetes can only be treated with insulin injections. Previously, cadavers and animal pancreas products were the primary sources. Both sources posed a risk of allergenic reactions, because insulin had to be purified from a complex mixture of proteins. There was also a potential for the transmission of disease, particularly viruses and especially of unknown viruses. Today, diabetics receive insulin, a recombinant protein produced in bacteria (*Escherichia coli*). Human insulin produced by recombinant technology has replaced bovine and porcine insulin preparations. The commonly available human insulins represent groups of short-, intermediate-, and long-acting insulins and biphasic mixtures. All groups have different characteristics with respect to the onset and duration of action. DNA recombination technologies provide the opportunity for the creation of insulin analogs with improved function. One group represents rapid-acting analogs known also as “rapid-onset” and “ultra-short-acting” insulins and includes *insulin aspart* and *insulin lispro*. Both recombinants contain modifications in the amino acid sequence of the insulin B chain. The advantage of using these analogs is a faster response in comparison with conventional short-acting insulin;

therefore, they can be injected immediately before meals or even after eating and are useful for young patients. The disadvantage of the rapid-acting analogs is the relatively short duration of insulin action. Another group of recombinant insulins are long-acting analogs that include *insulin glargine* (created by substitution and adding amino acids) and *insulin detemir* (created by adding a fatty acid chain to enhance binding to albumin). The advantage of the long-acting analogs is the more reproducible absorption and reduced risk of hypoglycemia (UBS Warburg Report, 2001).

1.8.2 Treatment of type 2 diabetes Type 2 diabetes patients has a wider range of treatment options in comparison with type 1 diabetes. Treatment of type 2 diabetes includes lifestyle modifications and the application of oral hypoglycemic agents or eventually insulin injections. Diet and exercise help type 2 diabetics to control their blood glucose, blood pressure, and blood lipid levels. Five groups of oral hypoglycemic agents are used in the treatment of type 2 diabetes: sulphonylureas, alpha-glucosidase inhibitors, prandial glucose regulators, glitazones (thiazolidinediones), and biguanides (UBS Warburg Report, 2001). Often a combination of different anti-diabetic drugs is used to increase the efficiency of therapy.

1.8.3 Side effects:

Hypoglycemia, or abnormally low blood glucose, is a complication of several diabetes treatments. It may develop if the glucose intake does not match the treatment. The patient may become agitated and sweaty and have many symptoms of sympathetic activation of the autonomic nervous system resulting in feelings similar to dread and immobilized panic. Consciousness can be altered, or even lost in extreme cases, leading to coma and/or seizures or even brain damage and death. In patients with diabetes, this can be

caused by several factors, such as too much or incorrectly timed insulin, too much exercise or incorrectly timed exercise (which decreases insulin requirements), or not enough food, or an insufficient amount of carbohydrates in food. In most cases, hypoglycemia is treated with sweet drinks or comestibles. In severe cases, an injection of glucagon (a hormone with the opposite effects of insulin) or an intravenous infusion of glucose is used for treatment, but usually only if the diabetic is unconscious.

Patients may experience blurred vision if they have had elevated blood sugar levels for a prolonged period of time and then have the elevated levels rapidly brought to normal. This is attributable to a shift of fluid within the lens of the eye. Over time, vision returns to normal. Other possible side effects that may occur include skin reactions (redness, swelling, itching or rash at the site of injection), worsening of diabetic retinopathy, allergic reactions, sodium retention, and general body swelling.

1.9 Strategies to cure diabetes

Currently, there is no permanent cure for diabetes. People with type 1 diabetes must take insulin several times a day and test their blood glucose concentration three to four times a day. Frequent monitoring is important because patients who keep their blood glucose concentrations as close to normal as possible can significantly reduce many of the complications of diabetes, such as diabetic retinopathy and heart disease that tend to develop over time. People with type 2 diabetes can often control their blood glucose concentrations through a combination of diet, exercise, and oral medication.

Transplantation of whole pancreas or islet cells is the best method at present for curing type 1 diabetes.

1.9.1 Transplantation of islets and whole pancreas

The most promising therapeutic option for the treatment of type 1 diabetes is the transplantation of insulin-producing cells. Several different cell sources are considered as successful replacement therapies. Transplantation of the whole pancreas or pancreatic islets can result in insulin independence, but problems related to graft rejections are reasons for the low efficiency of this therapy (Brendel et al., 1999). Islet allografts usually mismatch for at least one MHC antigen. Therefore, without immunosuppression, these allografts are rejected. Transplantation of new insulin-producing β -cells, in the form of the whole pancreas or isolated islets, in combination with a specific glucocorticoid-free immunosuppression regime (Edmonton protocol, Shapiro et al., 2000), has been shown to ameliorate the disease by eliminating the need for exogenous insulin and normalizing glycosylated hemoglobin levels (Shapiro et al., 2003; Kaufman and Lowe et al., 2003; Ricordi et al., 2003; Titus et al., 2000). However, freshly isolated islets from at least two immunologically compatible donors are necessary for the successful transplantation of adequate amounts of tissue. Because the number of human donors is limited, wide scale application of this therapy is not possible.

Use of xenogenic tissue, such as porcine islets, is an alternative option, but use of strong immunosuppressive drugs and the risk of retroviral infections create barriers for clinical applications (Butler, 1998). Therefore, other cell sources must be considered with respect to the generation of transplantable insulin-producing cells for a therapy of diabetes.

1.9.2 Transplantation of insulin-secreting cell lines

Insulin-expressing cell lines such as β -cell lines derived from insulinomas could represent an unlimited, easily available source of insulin-producing cells (Miyazaki et al., 1990, Dufayet de la Tour et al., 2001, Itkin-Ansari.P et al., 2001, Narushima et al 2005). Many different cell types including fibroblasts, muscle cells, keratinocytes, and hepatocytes have been transfected with the insulin gene. Unfortunately, most of the engineered non- β -cells produce and secrete only small amounts of insulin, and the insulin release is not adequately controlled by glucose levels. Despite strict selection procedures, the risk of tumorigenesis remains because of the possibility of epigenetic changes, chromosomal instability, or mutation during the culture of the immortalized cells.

1.9.3 Pancreatic progenitors as a source of islet cells

Pancreatic ductal epithelium, through sequential steps of differentiation, gives rise to all pancreatic endocrine cell types (Hellerstrom et al., 1984; Slack, 1995). There is clear evidence for the existence of pancreatic stem/progenitor cells in the ductal epithelium (Bonner-Weir and Sharma, 2002). The cultivation of mouse pancreatic epithelium under appropriate conditions results in the differentiation of functional endocrine cells secreting insulin in a glucose-dependent manner and the normalization of high blood glucose levels after implantation into diabetic mice (Ramiya et al., 2000). Similar results have been reported for human pancreatic ductal epithelial cells (Bonner-Weir et al., 2000; Gao et al., 2003). Exocrine tissue contains cells that can differentiate toward a pancreatic endocrine phenotype (Lipsett et al., 2002).

1.9.4 Embryonic stem (ES) cells as a source for islet cells

Stem cells are defined by their ability both to self-renew and to differentiate into specialized cells (Czyz et al., 2003). ESC have a virtually unlimited proliferation capacity and can differentiate into derivatives of nearly all lineages (Hoffman et al., 2005, Liew et al., 2005). Pluripotent ESC represent potentially unlimited source of pancreatic cells for regenerative therapies. However, so far, current techniques do not allow the generation of pure populations of somatic cells from ESC.

ESC could be cultivated and directed to differentiate into the insulin-producing islet cells of the pancreas. Insulin-secreting cells derived from ESC *in vitro* release insulin upon glucose induction (Soria et al., 2001) and are able to normalize glycemia in streptozotocin-induced diabetic mice (Soria et al., 2000). Under non-selective culture conditions, a small subpopulation of ESC spontaneously differentiates into pancreatic endocrine cells (Shiroi et al., 2002, Kahan et al., 2003). Islet like-clusters show regulated insulin release *in vitro* but fail to normalize high blood glucose levels in animal models (Lumelsky et al., 2000, 2001). Addition of growth inhibitors, cytokines, manipulation of the culture conditions and the use of transcription factors associated with the β -cell lineage (Hori et al., 2002, Kania et al., 2003, Kahan et al., 2003, Stoffel et al., 2004, Blyszczuk et al., 2003 and Miyazaki et al., 2004), have yielded promising results. Human ESC differentiate into insulin-producing cells under various culture conditions (Assady et al., 2001, Segev et al., 2004, Schuldiner et al., 2000, Yan Shi et al., 2005). Although the differentiated ESC are insulin-positive, insulin mRNA is inconsistently detectable and

weak, and C-Peptide and secretory granules have not been detected (Rajagopal et al., 2003).

Major obstacles remain to be overcome before insulin-producing cells derived in this way can be used therapeutically; typically, there is a mixed population of cells with only the minority producing insulin, the insulin content per cell is lower than that in adult human β -cells, the regulation of secretion is not fully physiological, the differentiated ESC survive poorly, and most studies have depended on mouse ESC.

1.9.5 Generation of islet-like cells from adult stem and progenitor cells

There are many attempts to derive insulin-producing cells from adult stem cells. Transplantation of splenocytes regenerate insulin-producing cells (Ryu et al., 2001, Kodama et al., 2003), recently Nishio et al., 2006, showed there is no evidence of splenocytes regenerate into insulin-producing cells. Liver oval cells have been transdifferentiated into glucose-responding insulin-producing cells (Yang et al., 2002, Zalzman et al., 2003). Adult intestinal epithelium after treatment with glucagon-like peptide-1 (GLP-1) develop into glucose-responsive insulin-producing cells, which after transplantation into diabetic mice reverse experimentally induced diabetes (Suzuki et al., 2003). In rodents, hemopoietic organs harbor cells that can also differentiate into functional pancreatic endocrine cells. (Janus et al., 2003, Hess et al., 2003, Zorina et al., 2003, Ryu et al., 2001, kodama et al., 2003, Kofman et al., 2004). BM cells can differentiate *in vitro* under controlled conditions into insulin-expressing cells (Jiang et al., 2002, Jahr et al., 2003). Such cells transplanted under the kidney capsule of diabetic animals have been demonstrated to regulate glucose levels in the blood (Dong-Qi Tang et al., 2004). However, recent reports also showed that the so-called, trans-differentiation

capacity of adult stem cells is rather limited (kirchhof et al., 2002), could not be repeated (Morshead et al., 2002, Wagers et al., 2002), or may be the result of cell fusions with endogenous cells (Alvarez-Dolado et al., 2003, Terada et al., 2002). Therefore, further detailed studies are needed to solve these questions.

1.10 Adult stem cells

An adult stem (AS) cell is an undifferentiated cell found among differentiated cells in somatic tissues or organ systems. AS cells are known to maintain and regenerate/repair tissues and organ systems and have been found in tissues characterized by extensive regeneration such as bone marrow (BM) (De Haan et al., 2002), skin (Watt et al., 2001), intestinal epithelium (Potten et al., 1998), and liver (Theise et al., 1999).

Hematopoietic stem cells (HSCs) in BM provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes (Hay et al., 1966), because circulating blood cells survive for only a few days or months. BM stromal cells or mesenchymal stem cells (MSC) can give rise to osteocytes, chondrocytes, adipocytes, and tenocytes (Pittenger et al., 1999). Neural stem cells in the brain can differentiate into its three major cell types: neurons, astrocytes, and oligodendrocytes (Copray et al., 2006). Intestinal epithelial stem cells in the lining of the digestive tract give rise to absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells (Quinlan et al., 2006).

1.10.1 Transdifferentiation

AS cells can be differentiated into cells not normally associated with their “committed” state (Fuchs et al., 2000). This property is known as transdifferentiation or plasticity.

Several reports have shown that hematopoietic stem cells from BM develop into neural, myogenic, and hepatic cell types, and neural or skeletal muscle stem cells develop into the hematopoietic lineage (Bjornson et al., 1997, Clarke et al., 2000, Fuchs et al., 2000, Galli et al., 2000, Gussoni et al., 1999, Jackson et al., 1999, Petersen et al., 1999). BMSC differentiate into cardio-myocytes (Fukuda et al., 2003), whereas mesenchymal stem cells develop into adipocytic, chondrocytic, or osteocytic lineages (Pittenger et al., 1999).

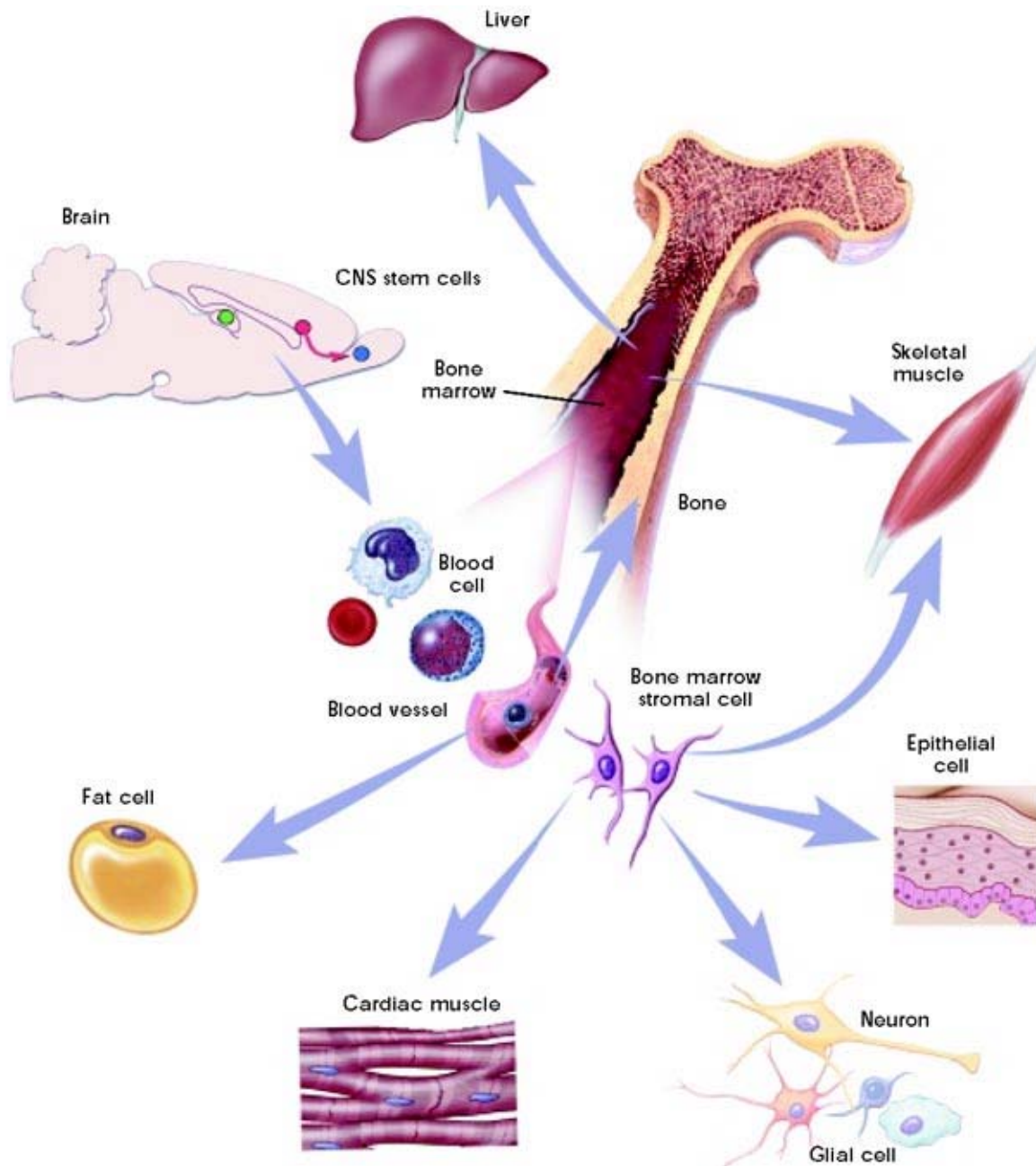


Figure 1.6 **Transdifferentiation of adult stem cells.** BM cells can differentiate into liver cells and skeletal muscle cells. BMSC can differentiate into cardiac muscle cells, neurons, epithelial cells, and skeletal muscle cells. Brain stem cells can differentiate into blood cells. (This figure is adapted from the website <http://stemcells.nih.gov/info/basics/basics4>.)

1.11 Chromatin remodeling of stem cells

The key goal of stem cell research is to attain an understanding of the manner in which differentiation is controlled and the direction that cellular differentiation will take. This should result in a better appreciation of stem cell biology and allow the optimal use of these cells in cell therapy.

Stem cell differentiation was controlled by both intrinsic regulators and the extracellular environment (niche). Under appropriate conditions in cell culture, stem cells can differentiate spontaneously. However, spontaneous differentiation is generally inefficient and leads to a heterogeneous population of differentiated and undifferentiated cells, which are not useful for cell-based therapy and also complicate biological studies of particular differentiation programs. Thus, stem cell expansion and differentiation *ex vivo* are generally controlled by “cocktails” of growth factors, signalling molecules, and/or genetic manipulation. Clearly, more efficient and selective methods are needed to direct the proliferation and the differentiation of stem cells, in order to produce homogeneous populations of particular cell types. This may not only be essential for the therapeutic use of stem cells, but will also greatly facilitate studies of the molecular mechanisms of development.

Actual stem cell fate decision might be governed by the expression patterns of transcription factors that may be under the control of methylation and deacetylation of chromatin, whereas commitment or differentiation might be triggered and regulated by external regulatory pathways, being activated by interactions of BM cells with growth factors or cytokines or the marrow microenvironment. Once these gene expression

patterns are known, small molecules might be identified that would favour their activation leading to the *in vitro* maintenance or expansion of not only BM stem cells but also possibly stem cells belonging to a variety of other somatic tissues.

2. Aim of the study

The major goal of this work was to define a protocol, required for the development of a cell therapy for diabetes in a mouse model.

1. The development of a reproducible culture protocol for generating insulin-producing cells by using bone marrow stem cells.
2. The evaluation of the contribution of chromatin reprogramming to the *in vitro* differentiation of BM cells, with the use of the chromatin remodeling factors TSA, azacytidine, sodium butyrate and DMSO.
3. The histological evaluation of differentiated cells for the expression of pancreas-specific hormones.
4. The analysis of endocrine pancreas-specific transcription factors and genes during development.
5. The functional analysis of differentiated cells, specifically with respect to glucose-regulated insulin release.

3. Material and Methods

3.1 Chromatin remodeling factors

3.1.1 Trichostatin A

Molecular Formula: $C_{17}H_{22}N_2O_3$

Formula weight: 302.4 Dalton

Trichostatin A (TSA; 4, 6-dimethyl-7-[p-dimethyl-aminophenyl]-7-oxohepta-2,4-dienohydroxamic acid) is an inhibitor of histone deacetylase (HDAC; including the mammalian form), isolated from *Streptomyces*. It serves as an antifungal antibiotic and inhibits the eukaryotic cell cycle at the beginning of the growth stage. TSA can be used to alter gene expression by interfering with the removal of acetyl groups from histones and therefore alters the ability of DNA transcription factors to access the DNA molecules inside chromosomes. Thus, TSA has uses as an anti-cancer drug. By promoting the expression of apoptosis-related genes, it may lead to cancerous cells surviving at lower rates, thus slowing the progression of cancer.

3.1.2 Dimethyl sulfoxide (DMSO)

Molecular Formula: C_2H_6OS

DMSO is an amphipathic molecule and is one of the most commonly used chemicals in the biological and medical sciences as a solvent for water-insoluble substances and a cryopreservant for various cell lines including ES and adult stem cells. It has multiple

effects on cellular functions such as metabolism and enzymatic activity and on cell growth by affecting cell cycle and apoptosis (Trubiani et al., 1999). It also changes cell fates by inducing the differentiation of various types of cells (Geder et al., 1987, Oh et al., 2004). Moreover, DMSO effects DNA methylation status (Iwatani et al., 2006).

3.1.3 Sodium butyrate

Molecular formula: $\text{CH}_3\text{CH}_2\text{CH}_2\text{COONa}$.

Formula Weight: 110.09 Dalton

Sodium butyrate, an HDAC inhibitor, significantly enhances survival and reduces both neuropathological effects and motor deficits in R6/2 transgenic mouse models. Sodium butyrate and similar HDAC inhibitors are currently being tested as anticancer drugs because of their inhibitory effects on cell proliferation (Kuefer et al., 2004). Sodium butyrate/retinoic acid co-stimulation induces apoptosis-independent growth arrest and cell differentiation (Buommino et al., 2000).

3.1.4 Azacytidine

Molecular formula: $\text{C}_8\text{H}_{12}\text{N}_4\text{O}_5$

5-Azacytidine or 5-aza-2'-deoxycytidine is a chemical analog of cytosine nucleoside present in DNA and RNA. Cells in the presence of 5-azacytidine incorporate it into DNA during replication and into RNA during transcription. The incorporation of 5-azacytidine into DNA or RNA inhibits methyltransferase thereby causing demethylation in that sequence and affecting the way that cell regulation proteins are able to bind to the DNA/RNA substrate. It is also used in the treatment of myelodysplastic syndrome.

3.2 Mice

Female mice (6-10 weeks old, C57BL/6) were purchased from Harlan (HARLAN WINKELMANN GmbH, Borchon, Deutschland, www.harlan-winkelmann.de) and maintained in a specific pathogen-free facility until used. Mice were treated in accordance with institute guidelines.

3.3 Isolation of mouse bone marrow cells

Mice were sacrificed by cervical dislocation. Bone marrow (BM) was flushed out from the tibias and femurs of C57BL/6 mice with Dulbecco's modification of Eagle's liquid medium (DMEM; ICN) in the presence of 10% fetal bovine serum (FBS) by using a 27 Gauge needle. Cells were pelleted after centrifugation at 800 rpm for 10 min at room temperature (RT), the supernatant was discarded, and the pellet was resuspended in an appropriate volume of medium.

3.4 Culturing and differentiation of BM stem cells

The BM cells were plated at a density of 1×10^6 cells/cm² in 6-well plates in normal DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 U/ml glutamine and allowed to adhere to the cell culture plate. After 12 hrs of incubation, nonadherent cells were washed out with fresh serum-free DMEM. The cells were treated with TSA/butyrate/azacytidine/DMSO (kindly donated by Prof. Dr. Jürgen Bode, GBF, Braunschweig) at 55 nm/ml in serum-free DMEM for 3 days. For the induction of differentiation, chromatin remodeling factors were withdrawn from the medium, and the cells were then cultured for an additional 7 days in specific induction medium containing

a 1:1 ratio of DMEM:DMEM/F12 (GIBCO) supplemented with 10% FBS and 10 nm/ml glucagon-like peptide-1 (Glp-1), in the presence of high (25mM) glucose. The medium was changed every 2 days according to the cell requirements.

3.5 Isolation of pancreatic islets

Pancreatic islets from mice were isolated according to a previously described method with a few modifications (Gotoh et al., 1985). Briefly, 6- to 10-week-old female C57BL/6 mice were killed by cervical dislocation. The pancreata were removed, without ductal injection or distention, cut into small pieces, and washed three times with ice-cold Hank's balanced salt solution (HBSS), (GibcoBRL, Life Technologies, Cat. No.14185-045) in order to remove released trypsin. The resulting pieces were suspended in 5 ml HBSS, containing 2,500 U collagenase Type 2 (Worthington Biochemical Corporation, Cat. No. 9001-12-1) and digested with shaking at 37°C for 30 min. The digested sample was washed three times with cold HBSS. The pellet was suspended in 10 ml 25% Ficoll solution (1.084 g/ml); the three uppermost layers consisted of 5 ml 23% (density 1.077g/ml), 5 ml 20% (density 1.068g/ml), and 5 ml 11%(density 1.038g/ml) Ficoll solution, respectively. Islet purification was performed by centrifugation at 800 g for 15 min at 4°C. Islets were harvested from the interfaces of the 11% and 20%, 20% and 23%, and 23% and 25% Ficoll solutions and washed with cold HBSS. Islet cell viability was confirmed by trypan blue exclusion.

3.6 Dithizone staining

Abbreviation: DTZ (Merck; Whitehouse Station, NJ)

Chemical Formula: $C_{13}H_{12}N_4S$

IUPAC Name: Anilinoimino-phenylazo-methanethiol

Preparation of stock solution: Dissolve 50 mg Dithizone in 5 ml DMSO. Store at – 20°C until used.

Preparation of working solution: Bring stock solution to RT. Filter using 0.2 µm nylon filters.

Staining method: Remove excess medium from the tissue culture plate containing differentiated BM cells. Add 10 µl working DTZ solution to the 1 ml culture medium. Incubate the tissue culture plate at 37°C for 30 min in DTZ solution. Rinse the cells three times with HBSS. Observe the crimson-red-stained clusters under a stereomicroscope (Zeiss Axiovert S100). After examination, refill the tissue culture plate with DMEM containing 10% FBS. Stain completely disappears after 5 hrs of incubation with DMEM containing 10% FBS.

3.7 Newport Green DCF labeling

Abbreviation: NG

NG is an ester (diacetate form, NG-Ac; Molecular Probes Europe; Leiden, The Netherlands).

Molecular formula: $C_{43}H_{30}Cl_2N_4O_8$

Molecular weight: 801.64

Principle: The ester form (NG-Ac) was used for fluorescence studies on living cells. NG-Ac is cell-permeant and therefore diffuses across cell membranes. Once inside the cells, this ester is cleaved by intracellular esterases to yield a cell-impermeant fluorescent indicator able to bind to zinc. The NG indicator exhibits an increase in fluorescence emission on binding to Zn, with a slight shift in wavelength; single excitation and emission spectral peaks were measured at wavelengths of 485 and 530 nm, respectively.

Method of labeling: Differentiated BM cells cultured in Tissue Tek chambered slides washed with phosphate-buffered saline (PBS) three times. Cells in PBS were exposed for 30 min at 37°C to 25 mM NG-Ac containing 1.5 µl/ml Pluronic F127 (Molecular Probes) to enhance the penetration of the probe. Cells were rinsed with PBS thrice and analyzed using confocal microscope.

3.8 Immunocytochemistry

Immunofluorescence analysis allows the investigation and characterization of BM cell differentiation in terms of the proteins expressed, at both the intracellular (e.g., intermediate-filament proteins) and extracellular (e.g., cell-surface antigens) levels. The method is especially valuable in BM cell differentiation studies, because differentiation of stem cells often results in a heterogeneous population. Immunofluorescence allows the detection of even a small minority of antigen-positive cells against a largely negative background. Two methods fixation were used (i) methanol:acetone (suitable for cytoskeletal proteins, such as intermediate filaments) and (ii) paraformaldehyde (suitable for intracellular proteins).

Reagents and equipment

Paraformaldehyde fixation solution: 0.4 g paraformaldehyde and 10 ml PBS mixture heated at 60°C; stirred until the solution becomes clear, and then cooled to RT (prepare shortly before use).

Preparation of 10% serum solution: 1 ml heat-inactivated serum mixed in 9 ml of PBS and store at 4°C. (Do not use serum from the same species as has been used for the preparation of the primary antibody).

Preparation of sodium citrate buffer:

(10 mM sodium citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate)	2.94 g
Distilled water	1,000 ml

2.94 g of Tri-sodium citrate was dissolved in 1000 ml distilled water completely. pH was adjusted to 6.0 with 1 N HCl and then 0.5 ml Tween 20 was added and mixed well. Sodium citrate buffer was stored at RT for 3 months or at 4°C for longer storage.

DAPI (4,6-diamidino-2-phenylindole) solution (Sigma): 1:500 dilution was prepared in PBS and stored in the dark at 4°C.

Fluorescence or Confocal microscope: The model LSM META510 confocal scanning laser system, including an Axiovert 200 M microscope (Carl Zeiss, Germany).

Fixation of differentiated cells: Cells cultured in Tissue Tek chamber slides (for immunocytochemistry) were fixed with 4% paraformaldehyde for 20 min at RT.

Staining differentiated cells: The fixed cells in Tissue Tek chambered slides were washed with PBS, three times. The cells were permeabilized with Triton-X 100 (1%) for

5 min (10 ml PBS+100 μ l Triton-X 100) and then rinsed (2x3 min) with PBS. Cell membrane was polarized with 0.1% glycine for 5 min (100 mg glycine in 100 ml PBS) then washed (2x3 min) with PBS. To prevent unspecific binding, cells were incubated with 10% serum solution (apply ~100 μ l overlay per chamber, in a chambered slide) in a humidified chamber at RT for 30 min. 60-100 μ l of the primary antibody was prepared by dilution in PBS (the optimal dilution of each antibody was determined separately). Cells with the primary antibody were incubated in a humidified chamber at 4°C overnight. Cells were gently washed three times with PBS. 100 μ l of the secondary fluorescence-conjugated antibody was prepared by dilution in PBS (the optimal dilution for each was determined separately). Cells were incubated with 100 μ l secondary antibody at 37°C in a humidified chamber for 45 min. Chambered slides were rinsed three times with PBS at RT. Cells on chambered slides were incubated with 200 μ l DAPI solution for counter-staining the nuclei, in a humidified chamber at 37°C for 10 min. Chambered slides were rinsed three times with PBS at RT. Chambered slides were mounted on a clean slide by using mounting medium (Merck-Kaiser's glycerol gelatin, which contains phenol; Lot no. 1.09242.0100) and analyzed by using a fluorescence or confocal microscope (Primary antibodies, secondary antibodies, isotype controls used in the study were listed in Tables 3.1, 3.2, 3.3 respectively).

Staining of paraffin sections: Paraffin-embedded tissue sections were deparaffinized in two changes of xylene for 4 min each. Hydrated in two changes of 100% ethanol for 4 min each, followed by changes in 95% and 80% ethanol for 2 min each. Then rinsed in distilled water for 2 min. In a water bath, a staining dish was pre-heated containing sodium citrate buffer until the temperature reaches 98°C. Slides were immersed in the

staining dish and incubated for 60 min. The staining dish was removed from water bath and allowed the slides to cool for 20 min. The tissue sections were rinsed in washing buffer (2x2 min). The sections were blocked with normal serum-blocking solution for 30 min at RT. Serum was removed from sections carefully, taking care not to dry the section. Primary antibody was added at the appropriate dilution and incubated overnight at 4°C. Sections were rinsed in washing buffer (3x5 min). Secondary antibody was added and incubated for 1 hr at RT and then rinsed with washing buffer (3x5 min). Counter-stained with DAPI for 10 min and rinsed again with washing buffer three times. Mounted with mounting medium.

Staining cryo sections: Sections were brought to RT for 20-30 min. Sections were fixed with methanol (100%) at 4°C for 3 min and subsequently in acetone (100%) for 3 min at 4°C. Air-dried for 1 hr and rinsed with PBS for 10 min. The remaining steps were common for fixed cells and tissue sections-permeabilization, antibody incubation, and mounting techniques.

Table 3.1 **List of primary antibodies**

Antibody	Antigen	Company	Host
Insulin (H-86): sc-9168	aa 25-110	Santa Cruz Biotech	Rabbit
Insulin A (C-12): sc-7839	C-terminus of Insulin A chain	Santa Cruz Biotech	Goat
C-Peptide serum Cat. No. 4023-01	Purified synthetic	LINCO Research	Goat
Glucagon (C-18): sc-7779	C- terminus	Santa Cruz Biotech	Goat
Somatostatin sc-20999	aa1-116	Santa Cruz Biotech	Rabbit
PDX 1 (N-18) sc-14662	N- terminus	Santa Cruz Biotech	Goat
NGN3	Fusion protein	Prof. Michael S. German UCSF, California	Rabbit
Pax 6(H-295) sc-11357	aa 264-422 C-terminus	Santa Cruz Biotech	Rabbit

Table 3.2 **List of secondary antibodies**

Antibody	Antigen	Conjugate	Company	Host
Goat Ig G (C 2821)	Whole molecule	Cy3	Sigma	Rabbit
Rabbit Ig G	(H+L)	Alexa Fluor 488	Molecular probes	Goat
Rabbit IgG (C 2306)	F(ab') ₂ Fragment	Cy3	Sigma	Sheep
Rabbit IgG (A21206)	(H+L)	Alexa Fluor 488	Molecular probes	Donkey
Goat IgG-R (sc-2094)	Whole molecule	Rhodamine	Santa Cruz	Donkey

Table 3.3 **Isotype controls**

Antibody	Antigen	Company	Host
Rabbit IgG (111-001-008)	Fc fragment	Jackson Immuno Research	Goat
Goat IgG	Serum	Nordic immunological labs	Rabbit

3.8.1 **Confocal microscopy**

Immuno-labeled cell preparations were examined by fluorescence microscopy. Wherever possible, a confocal laser-scanning microscope (CLSM) was used instead of a conventional fluorescence microscope, as confocal images were considered to be superior. Furthermore, confocal microscopy allowed the co-localization of proteins, which was especially relevant to the three-dimensional structures that were observed with, for example, BM-derived pancreatic cells.

Confocal imaging was performed with an LSM META510 confocal scanning laser system on an Axiovert 200 M microscope (Carl Zeiss, Jena, Germany). The instrument settings are given below. Images were obtained with a Plan-Neofluar 40x/1.3 oil-immersion objective lens.

Table 3.4 **Parameters and settings for confocal microscopy**

Para- meters Fluorescent dye	Lasers	Excitation wavelength (nm)	Emission filter (nm)	Detector
Alexa 488/FITC	Argon (max.7%)	488	BP505-530	normal
Alexa 546/Texas Red/Cy3	HeNe1 (max.20%)	543	BP 560-615	normal
DAPI	Enterprise UV (max.14%)	364	BP 475-525	normal

3.9 Three-dimensional (3D) reconstruction image of differentiated cluster

The 3D projection and reconstruction were performed with the Projection in 3D view menu (projection method: transparent; rotation: along Y-axis) using LSM software.

Stack size:

X: 115.2 μm

Y: 115.2 μm

Z: 14.0 μm

Slice interval: $\frac{1}{2}$ μm

3.10 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a common immunoassay used for the detection and quantification of a substance based on immunological reactions. ELISA is a valuable tool because the technique is highly sensitive and specific and allows rapid and simultaneous processing of a large number of samples. This method was suitable for the quantification of secreted insulin from BM cell culture systems.

Equipment and reagents

Krebs Ringer Bicarbonate Hepes (KRBH) buffer composition: 118 mM sodium chloride, 4.7 mM

potassium chloride, 1.1 mM potassium dihydrogen phosphate, 25 mM sodium hydrogen carbonate, 3.4 mM calcium chloride, 2.5 mM magnesium sulphate, 10 mM Hepes, and 2 mg/ml bovine serum albumin.

Other reagents: D (+)-Glucose monohydrate (Cat. No. 1.08346.1000, Merck, Germany), DMEM (with 4.5 g/l glucose, Invitrogen), fetal calf serum (FCS; Gibco BRL, Cat. No. 011-06920), trypsin-EDTA solution (0.5% / 0.2% (w/v), ultrasensitive mouse insulin ELISA kit (Mercodia, Cat. No. 10-1150-01), Protein Bradford Assay (Bio-Rad Laboratories, Cat. No. 500-0006).

3.10.1 Quantification of secreted insulin: Medium was aspirated from 12-well tissue-culture plates containing BM-derived pancreatic islet-like cells and rinsed the cultures three times with 3 ml PBS. PBS was replaced with freshly prepared KRBH buffer supplemented with 3.8 mM glucose and incubated for 120 min at 37°C. KRBH buffer supplemented with 3.8 mM glucose was aspirated. KRBH buffer supplemented with

either 7 mM glucose, 12 mM glucose, or 27.7 mM glucose and 10 μ M tolbutamide (Sigma) was added to separate tissue culture plates and incubated for 180 min at 37°C. Supernatant was collected and stored at -20°C for determination of insulin release.

Secreted insulin assay procedure: Each determination was performed in duplicates for calibrators and unknowns. A calibration curve was prepared for each assay run. All reagents and samples were brought to RT before use. Calibrators used were 0, 0.025 μ g/l, 0.063 μ g/l, 0.22 μ g/l, 0.60 μ g/l, and 1.5 μ g/l. Unknown samples were added in triplicates. 50 μ l-enzyme conjugate was added to calibrators and unknown samples and incubated on shaker for 2 hrs at RT. After incubation plate was washed 6 times, and the reaction volume aspirated. 350 μ l wash buffer was added to each well, then aspirated completely, repeated 5 times. After the final wash, the plate was inverted and tapped firmly against absorbent paper. Substrate TMB (3,3',5,5'-tetramethylbenzidine) (200 μ l) was added to each well and incubated for 30 min on shaker. Stop solution (50 μ l) was added to each well. Plate was kept on shaker for approximately 5 sec for ensuring proper mixing of substrate and stop solution. The absorbance was measured at 450 nm and evaluated.

3.11 Intracellular insulin detection: Sufficient amount of trypsin-EDTA solution was added to cover the whole surface and incubated at RT for 2-3 min. Trypsin-EDTA solution was removed carefully and added 3 ml DMEM medium supplemented with 10% FBS. Cells were resuspended with a 2 ml glass pipette and transferred to 15 ml tubes and centrifuged for 5 min at 1,000 g at RT. Supernatant was aspirated, lysis buffer was added, then transferred to 1.5 ml tubes, and freeze-thawed in liquid nitrogen to break open the cell wall of the cells. Then centrifuged for 5 min at 13,000 g at RT. Supernatant was transferred to new 1.5 ml tubes and stored at -20°C for determination of intracellular

total protein and insulin levels. Secreted insulin level with ELISA and total protein content by using the Bradford assay were determined according to the manufacturer's recommendations.

3.11.1 Co-immunoprecipitation (Co-i.p)

Cell pellet was prepared by pipetting or by mechanically using a cell scraper. Cell pellet was freeze-thawed in liquid nitrogen to break open the cell wall and stored at -20°C , until processed. The pellet was thawed, 200 μl lysis buffer was added containing protease inhibitor and phosphatase inhibitor. All the steps were carried out on ice. The cells were lysed and the preparation was passed into labeled Eppendorf (eppi) vials. Then the vials were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was collected into a new eppi vial. 10 μl supernatant was taken into 50 μl of 2x SDS buffer and stored at -20°C . Anti-insulin antibody (1.65 μg) was added to the supernatant and incubated this mixture at 4°C on a rotating wheel for 2 hrs. After incubation, all the solution was brought to the base of the eppi vial by quick spinning. The sepharose beads were prepared by taking 300 μl original suspension of protein G – Sepharose 4 FF (Pharmacia, in 20 % ethanol) in 1.5 ml eppi vials. Beads were washed three times with 1 ml lysis buffer without protease and phosphatase inhibitor. 50 % of original suspension was resuspended in lysis buffer. 25 μl sepharose beads were added to each sample. Then bead/solution mixture was incubated for 1 hr at 4°C on the rotation wheel. After incubation, centrifuged them at 2,000 rpm for 2 min. The beads were washed seven times by using co-i.p buffer with protease inhibitor and phosphatase inhibitor, then resuspended in 20 μl 2x SDS without β -mercaptoethanol and stored at -20°C .

3.11.2 Western blot

Cell lysis: For 1 ml RIPA buffer, 1 μ l protease inhibitor was added. Scraped cells were added into 200 μ l RIPA buffer, cells were lysed by vortexing. The lysed cells were kept on ice for 30 min, and then centrifuged at 10,000 rpm, and the supernatant was collected for protein estimation.

RIPA buffer composition:

60 ml	10x PBS
6 ml	Igapan
3 gm	Sodium deoxycholate
0.6 gm	SDS

Made up to 600 ml with double-distilled water (DDH₂O)

SDS-Polyacrylamide gel electrophoresis

Separating gel (15%):

ddH ₂ O	3.6 ml
1.5 M Tris, P ^H -8.8	3.75 ml
Acrylamide (30:0.8)	7.5 ml
10% APS	100 μ l
TEMED	10 μ l

Stacking Gel:

ddH ₂ O	3.47 ml
1 M Tris, P ^H -6.8	625 μ l
Acrylamide	850 μ l
10% APS	50 μ l
TEMED	5 μ l

Method: The protein samples were resolved by gel electrophoresis. The transfer of the proteins was fractionated by SDS, was performed by using a semi-dry electroblotting system onto a nitrocellulose membrane. The membrane was blocked for 1 hr at RT, incubated with rabbit anti-insulin antibody (1:5,000) overnight at 4°C, washed in Tris-buffered saline-Tween (3x5 min each), and incubated with anti-rabbit horseradish peroxidase (1:10,000) for 1 hr at RT. The washing buffer was removed, and the membrane was prepared for chemiluminescence by using an ECL kit.

3.12 RNA extraction

Total cellular RNA was extracted from whole BM and differentiated cells by using TRIzol® (GIBCO/BRL). RNA extraction was performed as follows.

Samples were brought to RT. 133 µl 1-bromo-3-chloropropane (BCP) (Merck-Schuchardt) was added for every 1 ml sample. Centrifuged for 15 sec and waited for 10 min then centrifuged for 30 min, at 12,000 relative centrifugal force (rcf), at 7°C. Upper phase was collected carefully, without contaminating the middle or lower phases, into a fresh 1.5 ml eppi vial. Ice-cold isopropanol was added (slightly greater volume than that of the supernatant). All these steps were performed on ice. Samples were incubated at –70°C for 1 hr or use a prolonged incubation period of up to 2 days. The pellets were stored at –20°C. The samples were brought to RT. Immediately centrifuged for 30 min, at 12,000 rcf, at 7°C. Pellet were washed with 1 ml 75% ethanol for 20 min at 12,000 rcf. The pellets were dried at RT or at 37°C on a heater. The samples were dissolved in 7 µl water treated with diethylpyrocarbonate (DEPC water). Samples Kept at 55°C on a heater for 10 min. Centrifuged quickly, without opening the lid. 2 µl sample was taken a to

check the quality and quantity of RNA on a gel and by absorption. All samples were stored at -70°C .

3.13 Reverse transcription/polymerase chain reaction (RT PCR)

One criterion for establishing the successful differentiation of BM cells into pancreas cells *in vitro* is the expression of genes that are normally involved in pancreas development *in vivo*.

Reverse Transcription:

Reagents:

DEPC water

RNase inhibitor (Promega)

dNTP (Invitrogen)

Oligo dT primer (Invitrogen)

M-MLV reverse transcriptase (Promega)

Method: 2 μg RNA was taken for each sample in sterile tubes for (+) and (-) RT reactions. 1 μl oligo dT primer was added and made this preparation to 15 μl with DEPC water. Incubated for 5 min at 70°C , immediately placed on ice for 5 min, and then 24 μl reaction mix (see below) was added. A sufficient volume of the following (+)/(-) RT master reaction mixtures were prepared for all reaction tubes.

RT master reaction mixture

DEPC-water	13.4µl
MLV buffer	5.0µl
dNTP's	5.0 µl
RNAsin	0.6 µl
Total volume	= 24.0 µl

The samples were incubated for 2 min at 42°C, immediately 1 µl reverse transcriptase was added to the (+) RT samples, incubated for 1 hr at 42°C and then for 5 min at 95°C to denature reverse transcriptase.

PCR:**Reagents:**

Fast Start Taq DNA Polymerase (Roche)

PCR buffer (Roche)

Forward primer, 10 µM (Invitrogen)

Reverse primer, 10 µM (Invitrogen)

dNTP, 25 µM (Invitrogen)

DEPC water

Method: An aliquot of 1 µl each cDNA sample was taken into a sterile PCR tube. A sufficient volume of the PCR master reaction mixture was prepared for all reaction tubes and then 24 µl of this mixture was added to each tube.

PCR master reaction mixture

PCR buffer	2.5 µl
dNTP	0.5 µl
Forward primer	0.62 µl

Reverse primer	0.62 μ l
Taq DNA polymerase	0.2 μ l
DEPC water	19.55 μ l
Total volume	= 23.99 μ l

PCR cycling conditions

Cycles	Temp. (°C)	Time
1	95	4 min
	95	30 min
35	x ^a	30 min
	72	1 min
1	72	10 min

^ax is the annealing temperature dependent on the primer used.

The PCR products were stored at 4°C. These PCR products were checked on 2% agarose gel.

The oligonucleotide sequences specific for the selected genes involved in the development and function of pancreatic beta cells were presented in (Table 3.5).

Table 3.5 Primers for PCR

Gene	Primer sequence	Length of gene	Annealing temperature	No.of cycles
Glut2	5'ACAGAGCTACAATGCAACGTGG 5'CAACCAGAATGCCAATGACGAT	221 bp	53	35
IAPP	5'-TGATATTGCTGCCTCGGACC 5'-GGAGGACTGGACCAAGGTTG	233 bp	65	35

Isl-1	5'-GTTTGTACGGGATCAAATGC 5'-ATGCTGCGTTTCTTGTCTT	514 bp	50.2	35
Ngn3	5'-TGGCGCCTCATCCCTTGGATG 5'-AGTCACCCACTTCTGCTTCG	157 bp	60	35
Pax4	5'ACCAGAGCTTGCCTGGACT 5'CCCATTTCAGCTTCTCTTGC	236 bp	62	35
Pdx1	5'TGGATAAGGGAATTGCTTAACCT 5'TTGGAACGCTCAAGTTTGTA	582 bp	53	35
Hnf3 β	5'AGAAGCAACTGGCACTGAAGGA 5'GTAGTGCATGACCTGTTCGTAG	464 bp	60	35
Proinsulin I	5'GTTGGTGCCTTCCCTACCCCTG 5'GTAGAGGGAGCAGATGCTGGTG	300 bp	60	35
Proinsulin II	5'GTGGATGCGCTTCCTGCCCCTG 5'GTAGAGGGAGCAGATGCTGGTG	300 bp	60	35
Glucagon	5'CACTCACAGGGCACATTCACC 5'ACCAGCCACGCAATGAATTCCTT	221 bp	52	35
Pancreatic polypeptide	5'CTGCCTCTCCCTGTTTCTC 5'GGCTGAAGACAAGAGAGGC	337 bp	52	35
Somatostatin	5'GACCTGCGAACTAGACTGAC 5'TTTGGGGGAGAGGGATCAG	294 bp	52.2	35
M-Sur1	5'CCAGACCAAGGGAAGATTCA 5'GTCCTGTAGGATGATGGACA	236 bp	53	35
β -Actin	5'GTCGTCGACAACGGCTCCGGCATG TG 5'CATTGTAGAAGGTGTGGTGCCAGATC	220 bp	55	35

3.14 Electron microscopy

For post-embedding detection of insulin in differentiated cells, samples were fixed with 0.2% glutaraldehyde and 0.5% formaldehyde for 1 hr on ice, washed with PBS

containing 10 mM glycine, and dehydrated in a graded series of ethanol following the progressive lowering of temperature protocol (PLT method). Samples were then embedded in Lowicryl K4M (Polysciences, Inc, Warrington, USA, Cat. No. 15923) at -35°C and polymerized with UV-light (366 nm, 2 days at -35°C , 2 days at RT). Ultra-thin sections were cut with glass knives and collected onto formvar-coated copper grids (300 mesh). Sections were incubated with a rabbit polyclonal anti-insulin antibody (sc-9168, Santa Cruz biotechnology; 100 μg IgG protein/ml) overnight at 4°C , washed with PBS, and incubated with gold markers (protein A/G gold, 15 nm in diameter from Bio cell Cardiff, UK) for 30 min at RT. After being washed with PBS containing 0.01% Triton X-100 and in distilled water, samples were air-dried. Counterstaining was carried out with 4% uranyl acetate for 1 min. Samples were then examined in a Zeiss transmission electron microscope EM910 (Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV.

3.15 Nuclear imaging

Three-dimensional stacks of fixed cells were acquired with the Leica TCS SP2 confocal laser scanning microscopy (CLSM) (Leica Microsystems, Mannheim, Germany) using a 63x/1.32 NA oil immersion objective. A diode and an Argon laser were used for DAPI ($\lambda=405$ nm). For the analysis of the reversibility of the TSA-induced accessibility changes, BM cells without treatment were imaged before and again after TSA treatment with DAPI nuclear staining. For DAPI imaging, excitation was carried out with a laser diode at 405 nm, and emission was recorded at 420-480nm.

3.16 Microarray analysis

3.16.1 RNA isolation

Total RNA was isolated by using the Qiagen RNeasy column kit (Cat.No. 74104). Briefly, BM cells and differentiated cells at day 3 and day 10 were lysed with an appropriate volume of Qiagen RLT buffer. The samples were then homogenized, centrifuged with an equal volume of 70% ethanol, washed with Qiagen RW1 buffer for 15 sec, incubated with Dnase I for 15 min at RT, and washed sequentially with Qiagen RW1 and Qiagen RPE buffers. The RNA was eluted with 30 µl Rnase-free water and stored at -20°C until processed further.

3.16.2 DNA microarray hybridization

The quality and integrity of the isolated total RNA was checked by running all samples on 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). For biotin-labeled target synthesis starting from 3 µg total RNA, reactions were performed by using standard protocols supplied by the manufacturer (Affymetrix; Santa Clara, CA). Briefly, 3 µg total RNA was converted to double-stranded DNA by using 100 pmol T7T23V primer (Eurogentec; Seraing, Belgium) containing a T7 promotor. The cDNA was then employed directly in an *in vitro* transcription reaction in the presence of biotinylated nucleotides.

The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 12.5 µg each biotinylated cRNA preparation was fragmented and placed in hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix GeneChips for 16 hrs. After hybridization, the GeneChips were

washed, stained with Streptavidin Phycoerythrin (**SAPE**) solution and read on an Affymetrix GeneChip fluidic station and scanner.

3.16.3 Data analysis

Microarray data was analyzed by using the Affymetrix GCOS 1.2 and Data Mining Tool 3.1 software package. For normalization, all array experiments were scaled to target intensity of 150 or by using the default values of GCOS 1.2. The results were filtered.

3.17 Sources of chemicals

Chemical	Company
Acetone	Baker B.V., Deventer, Holland
Antibodies	Santa Cruz Biotechnologies
Collagenase Type 2	Worthington
DAPI	Sigma-Aldrich GmbH, Deisenhofen
DMSO	Sigma-Aldrich GmbH, Deisenhofen
EDTA	Sigma-Aldrich GmbH, Deisenhofen
Embedding medium OCT	Leica GmbH, Bensheim
Fetal bovine serum (FBS)	Invitrogen
Gelatine	Sigma-Aldrich GmbH, Deisenhofen
Glycin	Merck KG, Darmstadt
Glycerin gelatine, Kaisers	Merck KG, Darmstadt
HBSS	Sigma-Aldrich GmbH, Deisenhofen
H ₂ SO ₄	Sigma-Aldrich GmbH, Deisenhofen

Laminin	Sigma-Aldrich GmbH, Deisenhofen
NaCl	Merck KG, Darmstadt
Paraformaldehyde	Sigma-Aldrich GmbH, Deisenhofen
QIAquick PCR Purification Kit	Qiagen GmbH, Hilden
Rneasy Kit	Qiagen GmbH, Hilden
Triton X-100	Sigma-Aldrich GmbH, Deisenhofen
Tris-HCl	Sigma-Aldrich GmbH, Deisenhofen
Trizma Base	Sigma-Aldrich GmbH, Deisenhofen
Trypan blue	Gibco Life Technologies GmbH
Trypsin	Gibco Life Technologies GmbH
Tween 20	Sigma-Aldrich GmbH, Deisenhofen

Culture Media

Dulbeccos Minimal Essential Medium (DMEM)	Sigma-Aldrich GmbH, Deisenhofen
DMEM/Ham F12	Invitrogen

3.18 Statistical analysis

All values are given as means \pm standard error of the mean. When data between two groups were compared, an unpaired or paired Student's *t* – test was used. $P < 0.05$ was considered to be statistically significant for all comparisons.

4. Results

4.1 Chromatin remodeling factors induce differentiation of bone marrow cells

To evaluate the effect of chromatin remodeling factors on the differentiation of bone marrow (BM) cells into insulin-producing cells, the cells were treated with 55 nM TSA, 24 μ M azacytidine, 5 mM butyrate, or 1% DMSO. Murine BM cells were isolated, plated in 6-well tissue culture plates, and cultured in the presence of chromatin remodeling factors for 3 days and then maintained for an additional 7 days in differentiation induction medium containing 10 nM Glp-1 and high glucose (25 mM). After removal of TSA, cells cultured in the high glucose medium started to form spheroid-like cell clusters from day 5, attaining their maximum size and maximal number at day 10. This did not occur in control BM cells cultured in the absence of both TSA and high glucose (Fig 4.1.1A). Cells cultured in the presence of TSA for 3 days exhibited normal cell morphology (Fig 4.1.1B). The three-dimensional cellular morphology of the subsequently formed cell aggregations closely resembled pancreatic islet-like clusters (Fig 4.1.1C, 4.1.1D), as previously described (Ramiya et al., 2000, Yang et al., 2002). The islet-like clusters contain well defined edges and structure. As many as 300 to 350 clusters formed per well of 6-well culture plates. The cellular morphology indicated that BM cells

could differentiate into islet-like cells in the presence of TSA followed by high glucose culture.

The results of the experimental approach by using various chromatin remodeling factors inducing pancreatic islet-like clusters are summarized in (Fig 4.1.2). Sodium butyrate showed a similar effect to that of TSA but with fewer numbers of islet-like clusters being formed. Azacytidine was cytotoxic at higher concentrations but, at micromolar concentrations, was effective on BM cells, resulting in the formation of islet-like clusters. However, the number of clusters was relatively low. The number of cellular clusters obtained after treatment with sodium butyrate (200-250), azacytidine (150-200), or DMSO (around 300), and with TSA (300 to 350). Murine BMSC after treatment with sodium butyrate, azacytidine and DMSO differentiated into islet-like clusters, but with lower efficiency compared with TSA.

Expression of pancreas specific genes and transcription factors involved in the development of pancreas were evaluated by RT-PCR analysis. The culture treated with various chromatin remodeling factors and cultured in high glucose medium, expressed transcripts of insulin genes I and II and of transcription factors Isl 1, Pax4, Hnf3 β (Fig 4.1.3) after 10 days. Positive control was not shown in this figure, but it was presented in the figure with TSA treatment. All treatments stimulated the expression of pancreas specific genes.

As BM cells treated with TSA and subsequently cultured in the presence of high glucose showed a higher number and larger size of three-dimensional spheroid clusters in comparison with those seen after treatment with other chemicals, all further work was carried out with TSA.

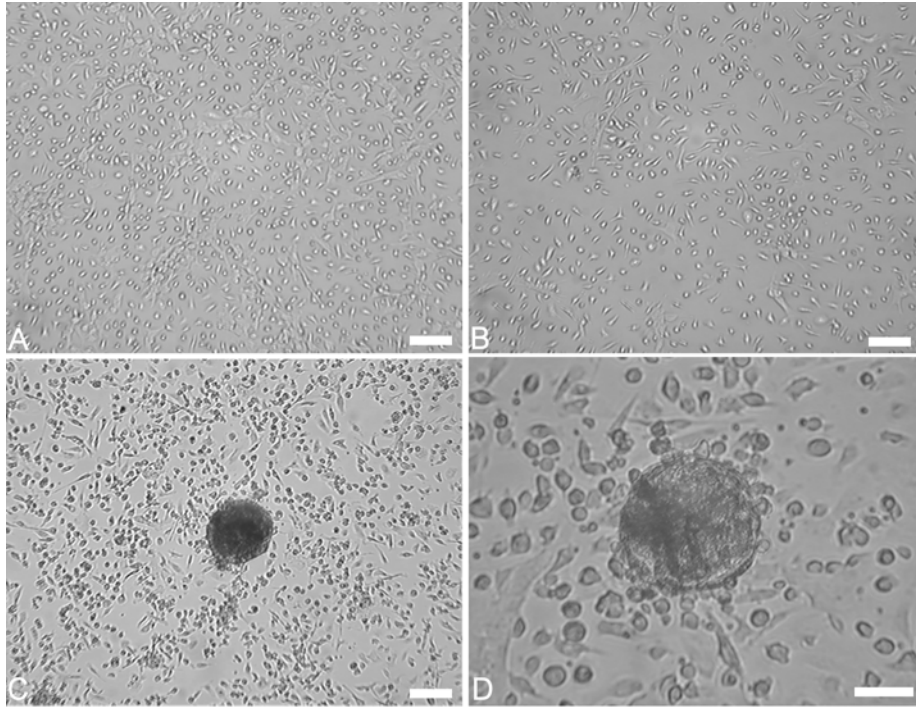


Figure 4.1.1 Chromatin-remodeling factor (TSA) induces bone marrow (BM) cells to differentiate into islet-like cell clusters

(A) BMSC cultured for 10 days in normal DMEM. BMSC were treated in serum-free medium for 3 days with histone deacetylase inhibitor TSA and then cultured in 10% FBS, GLP-1, and high glucose (25mM) for an additional 7 days. (B) BMSC cultured for 3 days in the presence of TSA. (C) BMSC cultured for 3 days in the presence of TSA and subsequently for an additional 7 days in high glucose medium, formed islet-like clusters (10x), at higher magnification (D), (40x).

Scale bar for (A), (B), (C) 100 μ m and (D) 50 μ m

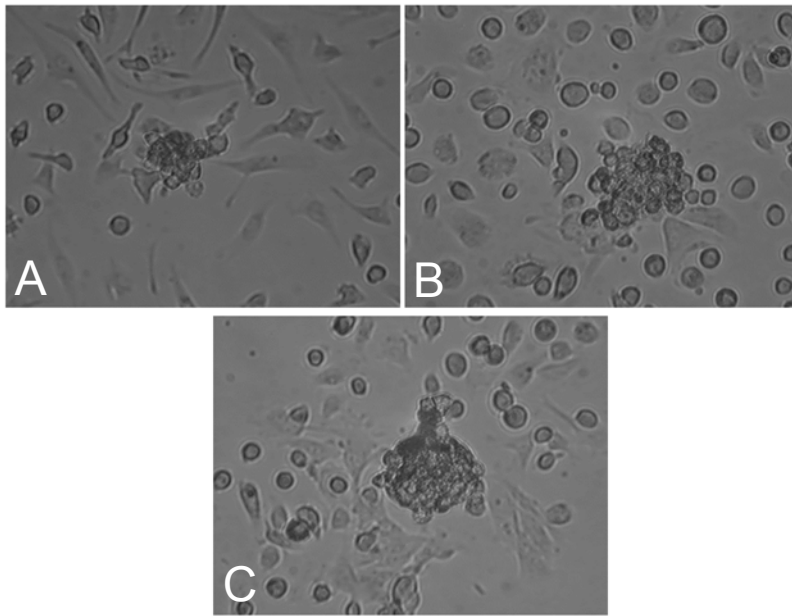


Figure 4.1.2 Formation of islet-like clusters after treatment with various chromatin remodeling agents.

BMSC were treated with chromatin remodeling factors for 3 days and then cultured in 10% FBS, GLP-1, and high glucose (25mM) for an additional 7 days formed islet-like clusters. After sodium butyrate treatment (A), Azacytidine treatment (B) and DMSO treatment, (C) (40x) BMSC differentiated into islet-like clusters. Scale bar for (A), (B), (C) 50 μm .

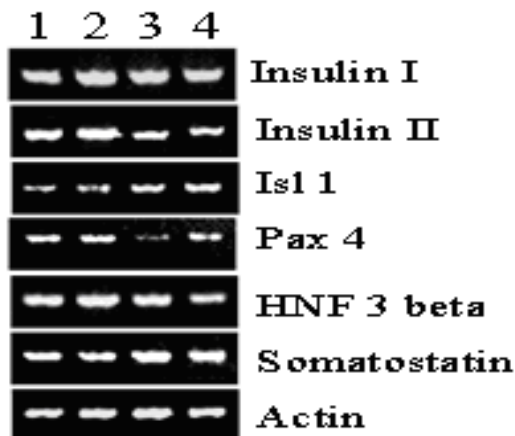


Figure 4.1.3 **Expression of pancreas specific genes after differentiation.**

After treatment with chromatin remodeling factors BMSC cultured in presence of high glucose for addition 7 days were analyzed for pancreas specific genes and transcription factors using RT-PCR.. Lane 1. Trichostatin A (TSA), Lane 2. Sodium butyrate, Lane 3. Azacytidine, Lane 4. DMSO

4.2 Detection of insulin containing cells in islet-like clusters

Zinc is required by pancreatic beta cells (β -cells) for the packaging of insulin, and free ionized zinc is present in the extra-granular space as a reservoir for granular zinc and forms an integral part of insulin crystals in the 2-Zn-insulin hexamer. We took advantage of these zinc pools to identify cells carrying out insulin synthesis in our cultures. The differentiated cells in cultures were stained with the zinc-chelating agent DTZ, and with the zinc-dependent fluorescent indicator Newport Green (NG-Ac).

We first determined whether isolated pancreatic islets from mouse were stained with DTZ and found that most of the islet cells did indeed stain crimson red. Differentiated

BMSC cells and, more specially, cells in the islet-like clusters exhibited distinct crimson red staining with DTZ (Fig 4.2A). Most of the crimson red stained cells are present in the core of islet-like cluster. Undifferentiated BMSC, cultured for 10 days in normal medium were not stained (Fig 4.2B).

In pancreatic β -cells insulin is packaged and stored in secretory granules, which accumulate in the cytoplasm. NG-Ac ester stains living cells, on enzymatic cleavage by cellular esterases, by binding to Zn^{2+} . Green fluorescent dots were seen in confocal images of differentiated cells stained with NG-Ac (Fig 4.2C). Individual cells revealed a heterogeneous intensity of fluorescence with cytoplasmic dots, suggesting the presence of insulin in secretory granules. The control BMSC cultures did not show significant staining with NG-Ac (Fig 4.2D). No nuclear staining was noted. Since insulin is expected to accumulate specifically in cytoplasm, this shows that there is no detectable non-specific staining.

These results indicate the presence of zinc in viable differentiated cells. The presence of cells positive for zinc-specific dyes such as DTZ and NG-Ac therefore suggested that insulin-producing cells could be derived from BM stem cells.

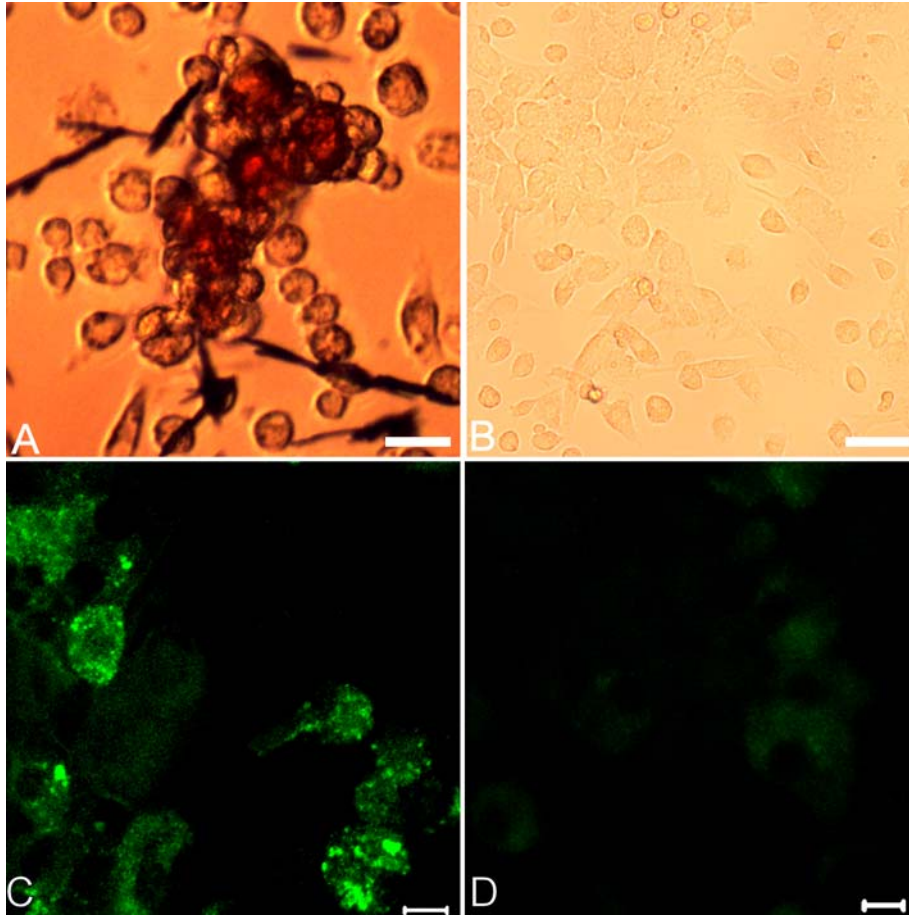


Figure 4.2 Detection of insulin-containing cells in islet-like clusters

Cells in islet-like clusters were stained with zinc-chelating agents, DTZ and Newport Green (NG-Ac). (A) Day 10: Cells distinctly stained crimson-red by DTZ are apparent in the differentiated islet-like clusters. (B) Undifferentiated BM cells (C) Individual differentiated cells stained with NG-Ac (D) Control cells for the NG-Ac stain. Scale bar for (A), (B) 20 μm and for (C), (D) 10 μm .

4.3 Detection of pancreas-specific hormones

To investigate the possible expression of pancreatic hormones in the cultured BM cells, immunofluorescence analysis was performed for insulin, C-Peptide, glucagon, and somatostatin. Immunostaining of the individual islet-like clusters, which were formed in BM cell cultures treated with TSA and subsequently cultured under high-glucose conditions, revealed large numbers of insulin-positive cells. Double-immunofluorescence analysis showed insulin (Fig 4.3.1A, left), C-Peptide (Fig 4.3.1A, middle), and co-labeling (Fig 4.3.1A, right) in the same cells of the islet-like clusters. Cells were counterstained by DAPI to reveal nuclei (Fig 4.3.1A). The co-localization of C-Peptide demonstrated the *de novo* insulin synthesis and excluded that cells only absorbed and concentrated insulin from the medium. Glucagon- and somatostatin-expressing cells were also present in the culture. The staining for glucagon was found in the periphery of the cell (Fig 4.3.1B), whereas somatostatin was dispersed in the cytoplasm (Fig 4.3.1C). Co-staining for insulin (Fig 4.3.1D, left) and somatostatin (Fig 3.3.1D, middle) showed that somatostatin-expressing cells were fewer in number than insulin-expressing cells in culture (Fig 4.3.1D, right). Transcription factor neurogenin 3 (Ngn3) was distinctly present in the nucleus of the differentiated islet-like cells (Fig 4.3.1E, middle and right). Matched isotype controls for rabbit IgG (Fig 4.3.1F) and goat IgG (Fig 4.3.1G) and without primary antibody (Fig 4.3.1H) served as negative controls. These results indicated that (1) differentiated BM cells synthesized insulin *de novo* as indicated by the presence of C-Peptide; similar to pancreatic β -cells. (2) other pancreatic-hormone-producing cells were also present in the culture after 10 days, interestingly higher number

of insulin expressing cells and (3) the transcription factor Ngn3 was expressed by the islet-like cells. Adult mouse pancreas was used as a positive control for the hormones insulin, C-Peptide, glucagon, and somatostatin (Fig 4.3.2). The transcription factor Ngn3 was only detected at 16.5 days in mouse embryo (Fig 4.3.2), also as a positive control.

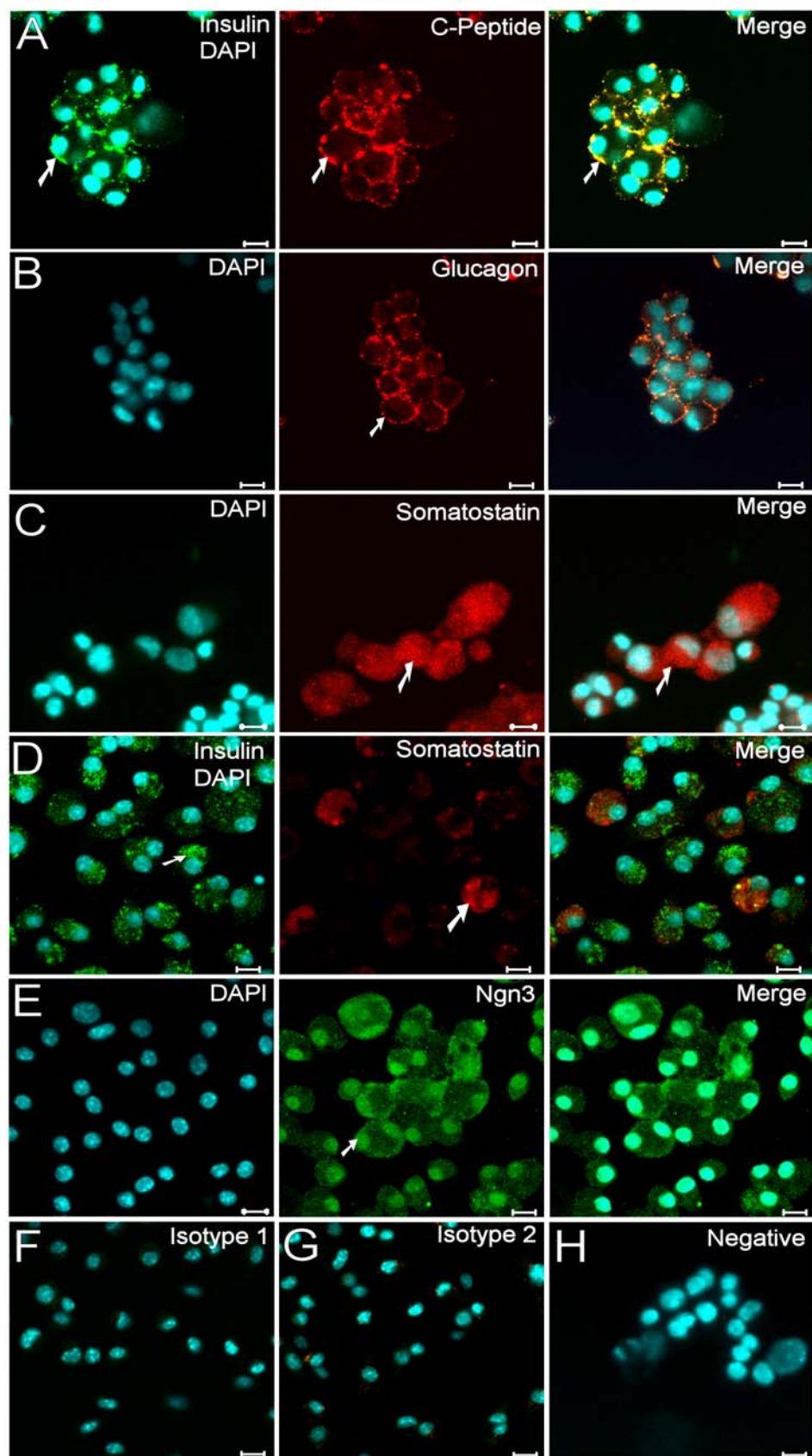


Figure 4.3.1 Detection of pancreas-specific hormones and transcription factors in differentiated BMSC

BMSC cultured for 3 days in presence of TSA and subsequent culturing for 7 days in presence of high glucose resulted in islet-like cultures. Immunostaining for pancreas-specific hormones in day 10 islet-like clusters. (A) Double-immunofluorescence analysis revealed insulin (green), C-peptide (red), and nuclear staining with DAPI (cyan); on merging both channels, the green (Insulin) and red (C-peptide) staining was seen as yellow. (B, C) Glucagon-positive cells (red) (B) and somatostatin-positive cells (red) (C) were present in the cultures (arrows). (D) Co-staining of insulin (green) and somatostatin (red) was observed. (E) Transcription factor Ngn3 was present in the nucleus of differentiated cells (arrow). (F-H) In the isotype control for rabbit IgG (Isotype 1; F) and goat IgG (Isotype 2; G) and in the negative control (no primary antibody; H), no immunostaining was observed; all were counterstained with DAPI (cyan) for nuclear staining. Scale bar 10 μ m.

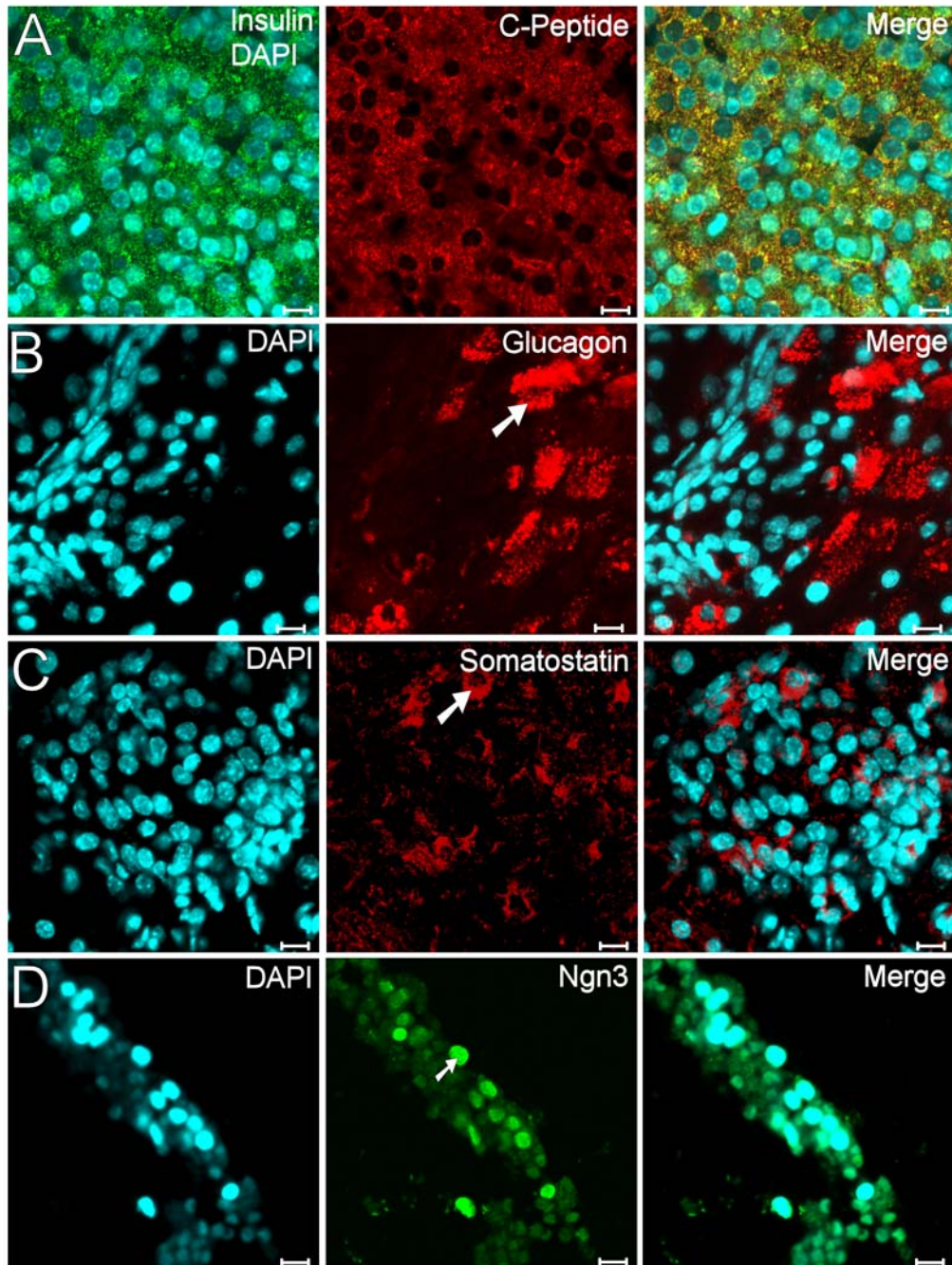


Figure 4.3.2 Specific hormone and transcription factor expression in mouse pancreas tissue

Specific hormone and transcription factors expression in mouse pancreas tissue were analysed by immunofluorescence. (A) Double-immunofluorescence analysis revealed β -cells containing

insulin (green) and C-Peptide (red), with nuclei stained by DAPI (cyan); on merging of both channels, the green (insulin) and red (C-Peptide) staining appears yellow. (B, C) Glucagon-positive (red) alpha cells at the periphery of an islet (arrow) (B) and somatostatin-secreting (red) delta cells also at the periphery of an islet (arrow) (C) in mouse pancreas. (D) Ngn3 antibody specific staining was present in the nuclei of 16.5-day mouse embryo pancreatic primordium (arrow). Scale bar 10 μm .

4.4 Three-dimensional (3D) reconstruction image of islet like cell cluster

Pancreatic β -cells synthesize insulin and store in secretory vesicles. Secretory vesicles are filled with insulin granules and C-Peptide, a by-product of insulin synthesis. Co-localization of insulin and C-Peptide immunostaining confirms the synthesis of insulin in differentiated BMSC. An islet-like cluster, three-dimensional (3D) reconstruction image was created by using confocal microscopy. The islet-like cluster was stained with anti-insulin (green) and anti-C-Peptide (red) antibodies. Various stacks were extracted from 3D image. The image shows perfect co-localization of insulin and C-Peptide (yellow colour) (Fig 4.4). Insulin and C-Peptide staining was granular, suggesting that insulin is located in secretory granules. Most of the insulin containing granules was situated near the periphery in the cytoplasm, without any background in the nucleus. This result is in agreement with the notion that the *in vitro* differentiated BMSC produce insulin and store it in secretory vesicles.

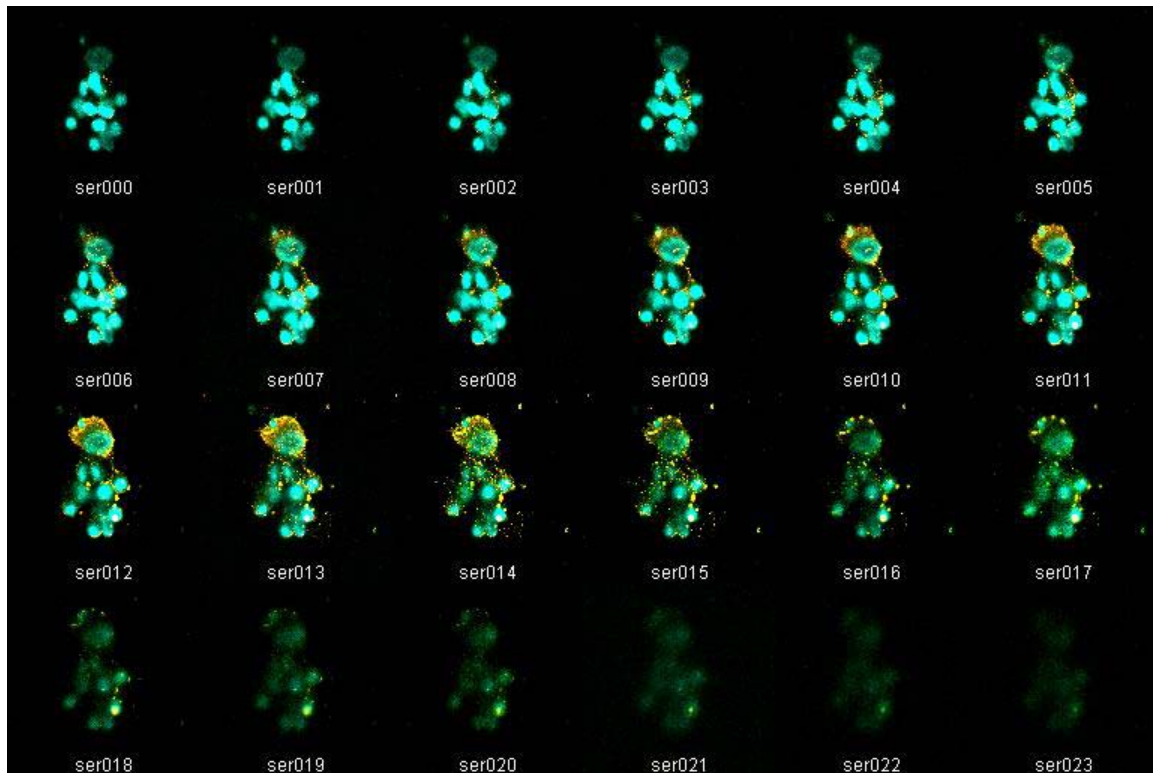


Figure 4.4 **Accumulation of insulin containing vesicles at the cell periphery**

Clusters of *in vitro* differentiated BMSC were stained with anti-insulin (green) and anti-C-Peptide (red) antibodies. The co-localization of insulin and C-Peptide (yellow dots) is appeared in the cytoplasm of islet like cell cluster. This image is visualized after extraction of different stacks of the 3D reconstruction image.

4.5 Glucose regulated insulin secretion from differentiated BMSC

In response to changes in the blood glucose concentrations insulin is released from pancreatic β -cells. To determine whether the differentiated BMSC were responsive to glucose concentrations, islet-like cells were exposed to different concentrations of glucose. Then insulin release upon exposure to high glucose was measured by using an ultra-sensitive mouse insulin ELISA. In order to enhance the sensitivity of these cells to

high-glucose challenge, the differentiated cells were switched to KRBH buffer containing 0.5% BSA and 3.8 mM glucose and incubated for 3 hrs. Then the differentiated BMSC stimulated by the addition of 7 mM, 12 mM, or 27.7 mM glucose to the KRBH buffer for 2 hrs in individual experiments. BM-derived islet-like cells after exposure to high glucose secreted insulin in a glucose-concentration-dependent manner (Fig 4.5.1). These data demonstrated that islet-like cell clusters derived from BM cells could secrete insulin in a glucose-regulated manner under the appropriate conditions as pancreatic β -cells.

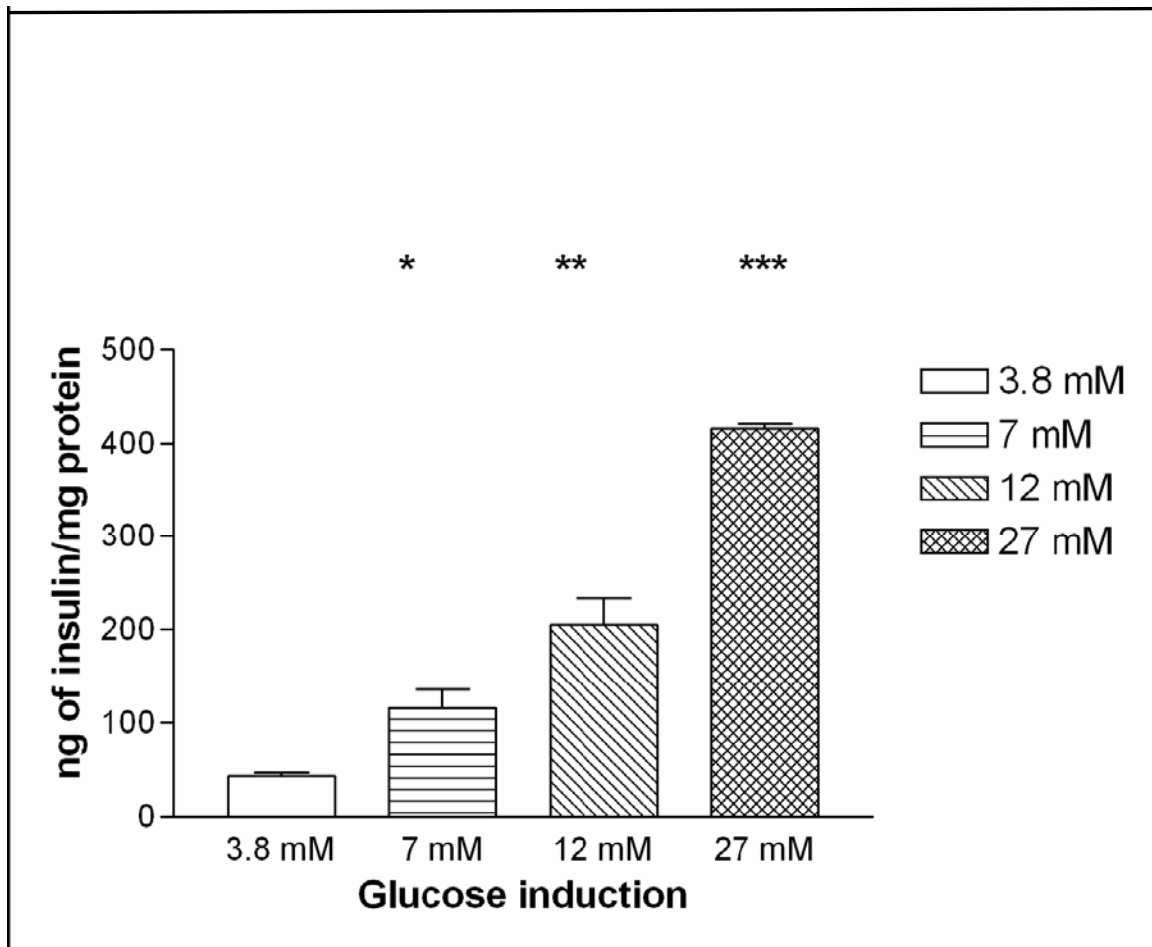


Figure 4.5.1 **Secretion of insulin upon high glucose challenge**

Differentiated BMSC were exposed to high glucose ELISA assay for insulin in Krebs Ringer Bicarbonate Hepes (KRBH) buffer removed from BM-derived islet-like clusters exposed to low (3.8 mM) and high glucose conditions (7 mM, 12 mM, and 27 mM) for 2 hrs.(omit to avoid repetition with text) Statistical significance was tested by Student's t test: * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Pancreatic β -cells synthesize and store insulin in the cytoplasm. BMSC treated for 3 days with TSA and BMSC differentiated in presence of TSA for 3 days and then additional 7 days in high glucose medium were taken for analysis of intracellular insulin. Cell lysates

of day 3 culture and day 10 cultures were subjected to immunoprecipitation and Western blot analysis. Cell lysates of differentiated BMSC contained stored insulin, as the cell lysate of day 3 cultures contained an immunopositive band, and the band intensity increased in cell lysate at day 10 (Fig 4.5.2). Pancreatic tissue served as a positive control. In contrast, no corresponding band was detectable in untreated BMSC (Fig 4.5.2). The data of insulin protein analysis therefore suggested that the differentiated islet-like clusters synthesized and stored insulin.

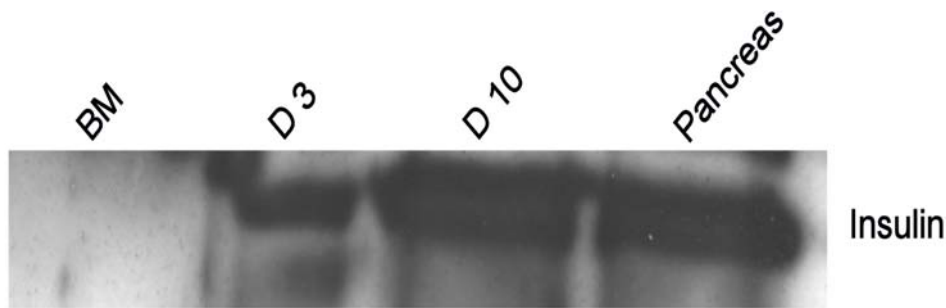


Figure 4.5.2 **Presence of intracellular insulin in differentiated BMSC**

BMSC were differentiated into islet-like clusters after treatment with TSA for 3 days and additional 7 days culturing in high glucose medium. The cell lysates of whole BMSC, 3 days culture, and 10 days culture were analysed for presence of intracellular insulin. Lane 1: Whole BM. Lane 2: Day 3 culture. Lane 3: Day 10 culture. Lane 4: Adult mouse pancreas (control).

4.6 Endocrine-specific gene expression in differentiated islet-like clusters

Pancreatic development and gene expression are regulated by specialized transcription factors. To determine whether endocrine-specific transcription factors and pancreas-specific genes were expressed during the differentiation into islet-like clusters, RT-PCR

analysis was performed at various time points during the culture of BM cells under high glucose conditions following TSA treatment. Transcripts for PDX-1, Pax4, Hnf3 β , and Isl 1 were not detectable in undifferentiated BM cells; in contrast, they were up-regulated in differentiated cells (Fig 3.5). PDX1 and Hnf3 β transcripts were expressed on day 3 after TSA treatment and increased by day 10. The PCR product of Ngn3 was visible in both undifferentiated and differentiated BM cells. PCR products for insulin genes I and II were visible at day 3 and day 10. In contrast, transcripts of other major islet-specific hormones, such as IAPP, glucagon, and somatostatin, were not detected on day 3 of differentiation and were expressed only on day 10. PCR products for the ATP-sensitive potassium (KATP) channel-specific sulfonylurea receptor (SUR-1) and glucose transporter gene (GLUT-2) were detectable only at day 10. Gene expression analysis in BM-derived islet-like clusters was similar to that in the native pancreas tissue and confirmed the differentiation of BM cells into islet-like cells *in vitro* upon treatment with TSA and subsequent culturing in high glucose.

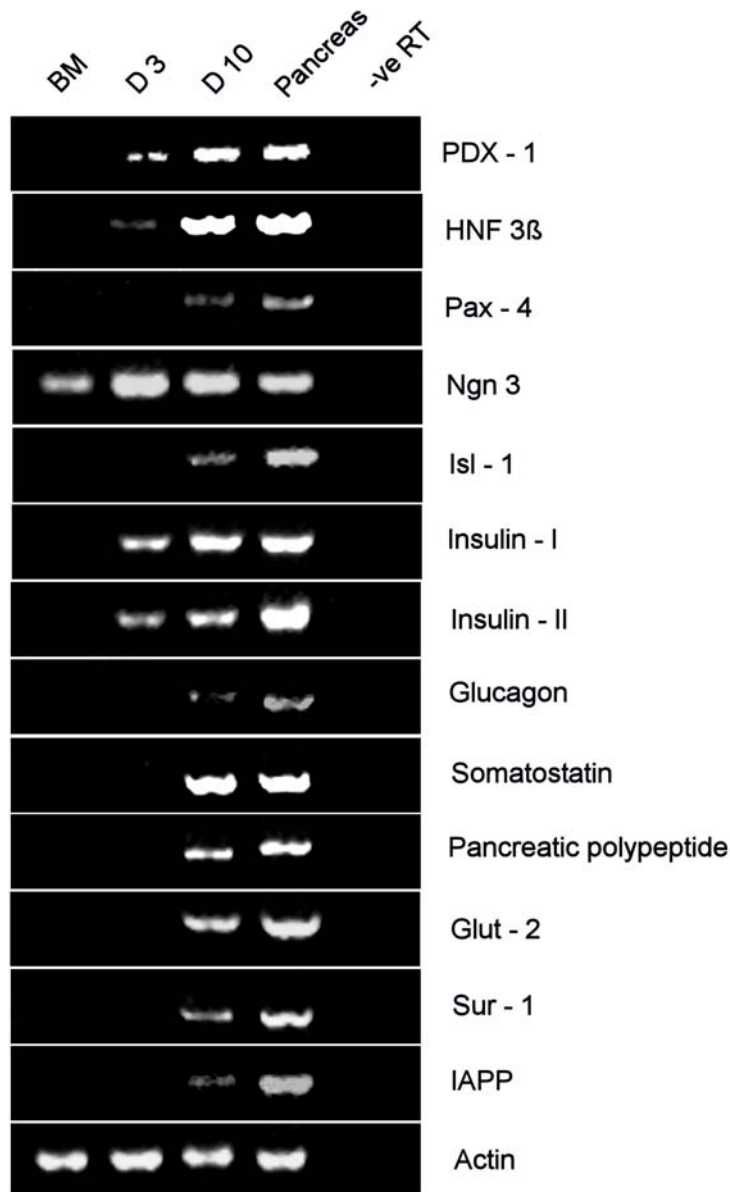


Figure 4.6 Endocrine-specific gene expression in differentiated islet-like clusters

BMSC differentiated into islet-like clusters after culturing in presence of TSA and high glucose medium. Total RNA isolated from whole bone marrow, day 3 culture, day 10 culture, adult mouse pancreas was subjected to RT-PCR analysis with primers for the indicated genes (listed right). BM: Undifferentiated whole BM. D3: Day 3 culture with TSA only. D10: Day 10 culture with high glucose and GLP-1. Pancreas: Adult mouse pancreas (positive control). Lane 5: No template (negative control).

4.7 Ultrastructural analysis of insulin-producing cell clusters

Ultrastructural analysis of BM-derived insulin-producing clusters was also performed. Differentiated cells at low magnification revealed structures typical of a secretory cell, with secretory vesicles containing dense granules (Fig 4.7A). Immunogold electron microscopy showed insulin within the small secretory vesicles of the insulin-producing clusters (Fig 4.7B). Gold-labeling also detected faint globular structures of differing size filled with a low-density material. Positive and negative controls for immunogold labeling are presented in Fig 4.7C and Fig 4.7D, respectively. Adult pancreatic β -cells showed several positive immunogold particles per secretory granule (Fig 4.7C). The pancreatic β -cells were examined only to validate the assay and not to correlate insulin-positive signals between the β -cells and the BM-derived insulin-producing clusters. Control BM cells showed no specific labelling, but a few non-specific particles were seen in the cytoplasm (Fig 4.7D). Thus, the differentiated BM cells exhibited features typical of an adult β -cell, and insulin granules were observed within the secretory vesicles suggesting that these cells had differentiated into pancreatic β -like cells capable of synthesizing insulin.

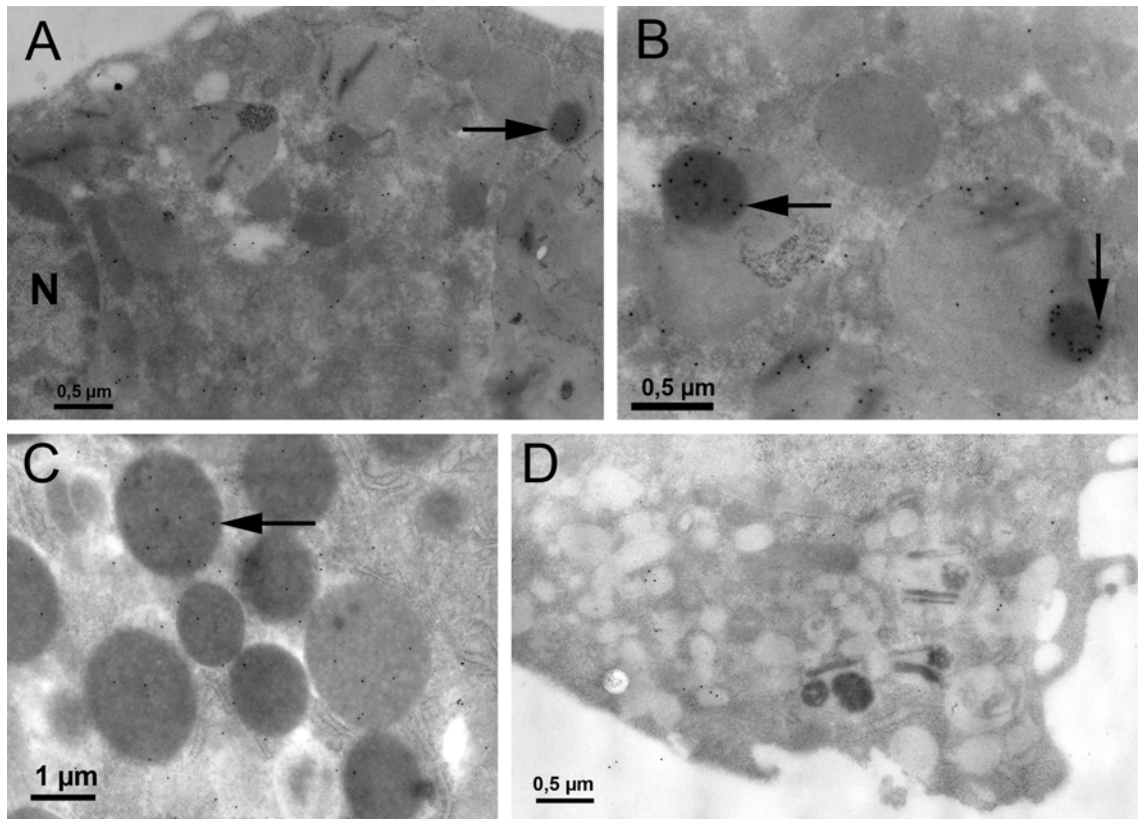


Figure 4.7 Presence of insulin granules in secretory vesicles

Differentiated BMSC were analysed for presence of insulin granules after post-embedding immunogold staining for insulin in day 10 islet-like clusters. (A) Secretory granules (arrow) are densely packed within the cytoplasm of the differentiated cell. (B) At higher magnification, insulin granules (arrows) are seen in secretory vesicles (black dots represent immunogold labeled insulin). (C) Positive control for immunogold labeling: positive granules (arrows) in adult mouse pancreas. (D) Negative control. Only non-specific staining is observed. Scale bar: (A) and (B) 0.5 μm, (C) 1 μm and (D) 0.5 μm.

4.8 Chromatin morphology after treatment with TSA

The chromatin morphology was studied before and after treatment of TSA. The chromatin accessibility changes following histone acetylation. Nuclei of control BM cells were seen as dense chromatin areas as brighter spots around the nucleolus and close to the nuclear membrane (day “0” Figure 4.8). After TSA treatment chromatin distribution was more homogeneous and most of dense chromatin disappeared (day “1” Figure 4.8). The chromatin decondensation attributable to acetylation is however reversible. These effects were well demonstrated in cultures of BM cells treated with TSA for three days, showed few cells with dense chromatin (day “3” TSA treated cell nucleus Figure 4.8), but majority of the cells chromatin still remain homogeneous. After a wash-out step all cells regained dense chromatin (day “5”, day “10” Figure 4.8). Regular condensed chromatin was visualized in all TSA untreated cells.

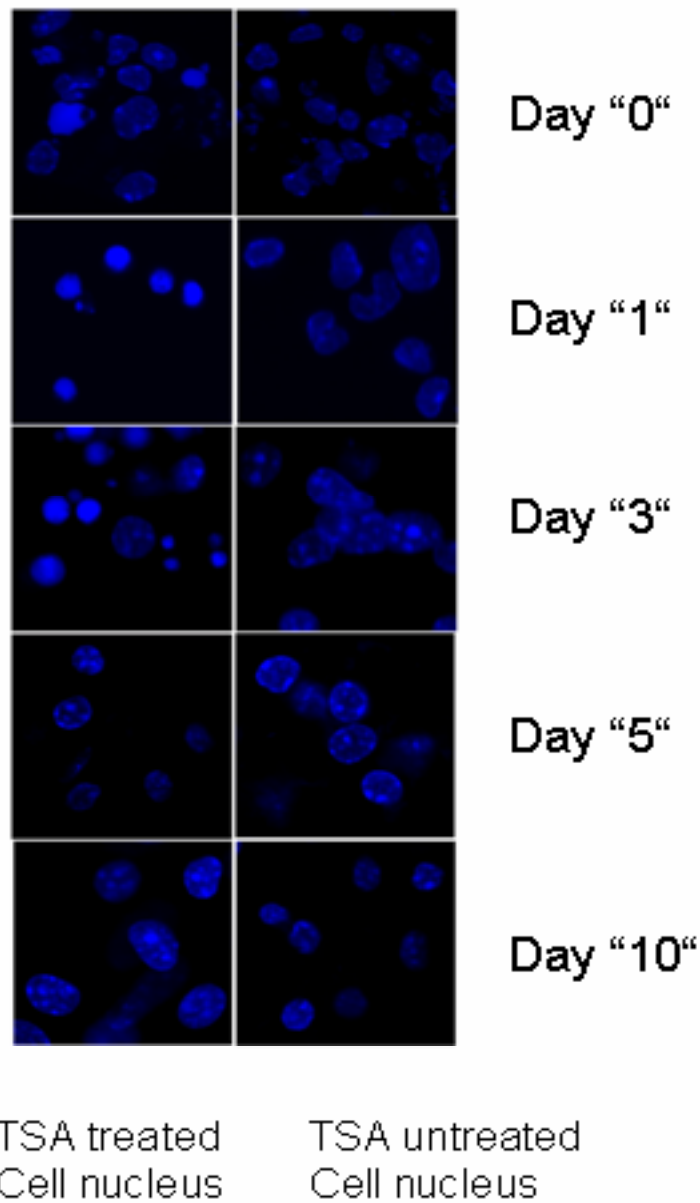


Figure 4.8 TSA-induced changes of interphase chromatin morphology.

Chromatin distribution in undifferentiated BMSC, after treatment with TSA and after differentiation into islet-like cells was analyzed. Nuclei of control cells contain dense chromatin areas, seen as brighter spots around the nucleolus and close to the nuclear membrane (day 0). TSA-treated cells exhibit a more homogeneous chromatin distribution, and most of the dense

chromatin disappears (day 1, day 3). The effect of TSA is reversible, since dense chromatin regions reappear after TSA is washed out (day 5, day 10 nuclei). The cells shown here were fixed and DAPI-stained to reveal the nuclei. Magnification 63x. (*In collaboration with Dr. Karsten Rippe, Heidelberg*).

4.9 Genes expressed at different time points of differentiation

The expression of genes at different time points during culture was analyzed by using microarray analysis. BM cells as a control and day 3 cultures and day 10 cultures after differentiation into islet-like clusters were taken for analysis. Control versus day 3 cultures revealed that 8964 additional genes were expressed, whereas control versus day 10 cultures showed the expression of 1076 additional genes. A comparison of day 10 with day 3 cultures demonstrated that an additional 160 genes were expressed in the older culture (Fig 3.8). These results indicated that BM cells that had been stimulated to differentiate into islet-like cells initially increased the numbers of genes that they expressed, although the number of active genes declined at later culture times.

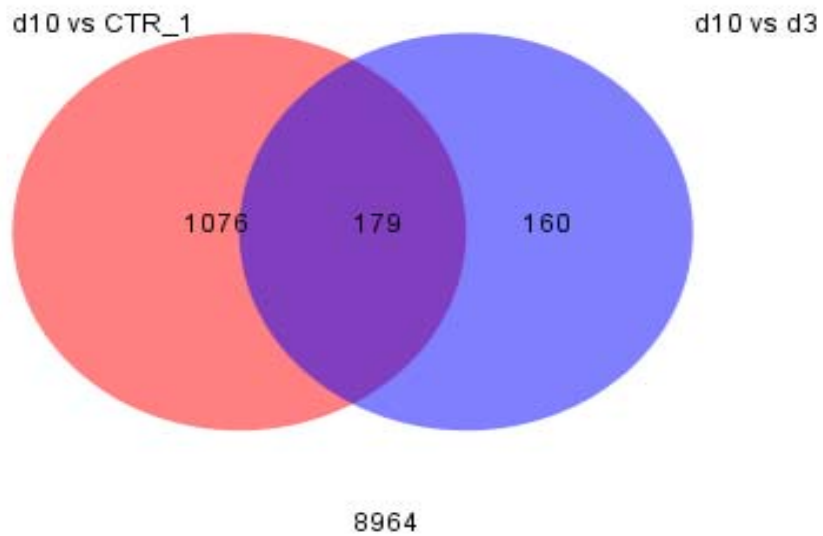


Figure 4.9 Analysis of genes expressed at various time points.

Control BM cells (CTR_1) versus cultures at day 3 (d3) and at day 10 (d10). Expressed genes were analyzed by microarray analysis. When BM cells were compared with to day 10 cultures, an additional 1076 genes were expressed (pink circle), whereas in the comparison of day 10 versus day 3 cultures, 160 additional genes were expressed (blue circle). A comparison of control BM cells versus the day 3 culture showed that 8964 additional genes were expressed.

5. Discussion

5.1 Chromatin remodeling factors induce differentiation of BMSC

Adult stem cells could potentially provide an abundant and ethically acceptable source of islet cells for transplantation therapies. Methods for stem cell differentiation must involve the activation of genes that are inactive, at least in part, because they lie in silent chromatin neighborhoods. Therefore chromatin reprogramming can contribute to the *in vitro* differentiation of BMSC into insulin-producing cells. We evaluated chromatin-remodeling agents if they could stimulate BMSC differentiation. TSA, azacytidine, butyrate, and DMSO are well-known chromatin-remodeling agents. BMSC were cultured in the presence of azacytidine, butyrate, or DMSO in individual cultures for 3 days, and after an additional 7 days in high glucose medium, we obtained low numbers of cellular aggregates. The three-dimensional cellular morphology of the cell aggregates closely resembled islet-like clusters. Sodium butyrate treatment resulted in a small number of islet-like clusters. Azacytidine was cytotoxic at higher concentrations but, at micromolar concentrations, exerted an effect on BMSC resulting in the formation of islet-like cells. DMSO treatment and high glucose medium also resulted in islet-like clusters.

The organ specificity of these clusters resulting from sodium butyrate, azacytidine, or DMSO treatment followed by culture in high glucose medium was confirmed by RT-PCR analysis by expression of pancreatic β -cell-specific genes, and the transcription factors necessary for development. The islet-like clusters expressed insulin gene I and II,

somatostatin, and the transcription factors Pax 4, HNF 3 beta, and Isl 1. The gene analysis showed that islet-like clusters resulting from the treatment of chromatin remodeling factors and high glucose medium expressed pancreas-specific genes. These results further suggested that sodium butyrate, azacytidine, or DMSO treatment leads to the differentiation of BMSC into a pancreatic β -cell-like population, but with low efficacy. Therefore culture conditions were established that allowed differentiation of BMSC into insulin. And there is a necessity to standardize the type and concentration of compounds with respect to the treatment and length of exposure and with regard to the subsequent culturing in high glucose and GLP-1-containing media.

BMSC that were treated with the histone deacetylase inhibitor (HDACi), TSA, for three days and subsequently cultured in high glucose medium (25mM) containing 10 nm GLP-1 produced large numbers of islet-like cell clusters. After 3 days of treatment with TSA, BMSC remain quiescent, without any multiplication. However, removal of TSA from the medium and exposure to high glucose conditions immediately resulted in the formation of cellular aggregates. Culturing BMSC for additional 7 days in high glucose medium increased the size and number of the cellular aggregates. Once these aggregates had achieved their maximum size in a time period of 10 days, no further growth occurred, and they remained static. The morphology of the cellular aggregates was similar to that of pancreatic islets, as previously reported. Islet-like clusters derived from BMSC appeared to contain clear margins. In contrast, BMSC cultured for 10 days in normal DMEM with 10% FBS showed normal morphology without any significant changes. These results thus demonstrated that BMSC treated with the HDACi, TSA, and cultured in high glucose medium differentiated into islet-like clusters. Further, BMSC treated with TSA and

subsequently cultured in the presence of high glucose showed a higher number and larger size of three-dimensional spheroid clusters in comparison with those seen after treatment with other chromatin remodeling agents. In this way concentration and incubation period were optimized and TSA was found to be optimal differentiation agent, therefore, all further work was carried out with TSA.

5.2 Detection of insulin-producing cells in the culture

BMSC treated with TSA and subsequently cultured in the presence of GLP-1 and high glucose medium produced islet-like clusters. To evaluate presence of insulin-producing cells in the culture, the cells were stained with Dithizone (DTZ) and Newport Green (NG-Ac), as pancreatic β -cells contain large amounts of zinc used in the synthesis, storage, and secretion of insulin.

DTZ is a zinc-binding substance, and pancreatic islets from animal species such as mouse, dog, pig, and human are known to be stained crimson red by its treatment, because of their higher zinc content compared with that of other tissues. DTZ staining is a simple and quick method specifically to identify insulin-producing cells from a mixed cell culture preparation of differentiated BMSC. Individual differentiated BMSC cells, specifically cells in the islet-like clusters, exhibited prominent crimson red staining upon staining with DTZ solution. In particular, cells present in the core of islet-like clusters stained crimson red; this is similar to the location of the position of pancreatic β -cells in the islets of Langerhans in which β -cells are present in the core surrounded by the mantle of other pancreatic hormone-producing cells. BMSC cultured for 10 days in normal medium showed no significant staining, whereas islet-like cells stained crimson red for DTZ indicating the presence of zinc-containing cells in the culture.

Newport Green (NG-Ac) is a zinc fluorescent probe; the loading of the esterified form of NG-Ac is reported to be easy, rapid, specific, and nontoxic to insulin-producing cells. NG-Ac produces fluorescence upon binding with zinc; therefore it is useful for the purification and collection of insulin-producing cells. The examination of the distribution NG-Ac inside the differentiated BMSC by confocal microscopy highlighted intense NG fluorescence mainly associated with secretory granules, possibly demonstrating that NG was competing with insulin for zinc inside these granules. Staining of individual cells revealed a heterogeneous intensity of fluorescence with cytoplasmic dots.

Positive cells for NG-Ac and DTZ staining thus demonstrated that insulin-producing cells were present in the differentiated BMSC culture. The use of NG-AC may be helpful for the purification and sorting of insulin-producing cells from *in vitro* differentiated BMSC for further use of these cells *in vivo*. These results strengthen the view that the *in vitro* differentiated cells in the islet-like cluster produce insulin.

5.3 Presence of transcription factors and genes specific for pancreas

To further characterize the islet-like cells derived from BMSC after treatment with TSA and high glucose, we analyzed the gene expression of a variety of endocrine pancreatic markers and transcription factors involved in the development of pancreas by using RT-PCR analysis. The differentiated cells expressed mRNA of the endoderm-specific and pancreas-specific transcription factor genes, HNF3 beta and pancreatic duodenal homeobox 1 (PDX1), respectively, and of the genes for insulin I, insulin II, glucagon, PP, and GLUT 2. The transcription factor, PDX1 expressed during day 3 of culture may have initiated a cascade of events leading to insulin transcription. High levels of PDX 1

transcript were found at day 10. Pax 4 transcripts were up-regulated at day 10; *in vivo* Pax 4 is essential for proper β -cell development, and its expression is restricted to endocrine progenitor cells. The neurogenin 3 (NGN3) transcript was highly expressed from the beginning; transcription factor NGN3 is involved in the proliferation and specification of early endocrine progenitors. GLUT 2 is a highly specific pancreatic β -cell or islet gene and plays an important role in glucose-stimulated insulin secretion. GLUT 2 expression was found in day 10 differentiated islet-like clusters. Transcript for the component of K^{+}_{ATP} channel, SUR 1, was also detected. Transcription factor Isl-1, which controls cell-fate decisions required for the differentiation of islet cells is expressed during BMSC differentiation. Further evidence for the activation of a pancreatic differentiation program in BMSC on following our protocol is the up-regulation of IAPP, a peptide almost exclusively expressed in β -cells and co-secreted with insulin. The transcripts of the major pancreas-specific hormones insulin I and insulin II, glucagon, somatostatin, and PP were also present in our cultures. The glucagon was gene expressed only at low levels; this correlates with the immunofluorescence analysis showing that only a few cells were positive for glucagon. This gene expression pattern was similar to that of mouse pancreas tissue. The analysis by RT-PCR thus demonstrated that, using TSA and high glucose culture conditions during differentiation increased the expression of pancreatic genes. Therefore, BMSC differentiated islet-like cells are similar to pancreatic endocrine cell population. Whole bone marrow, day 3 culture after TSA treatment and day 10 culture after high glucose and Glp-1 treatment were analyzed by microarray. Microarray analysis showed that a wide variety of genes were induced upon treatment of BMSC with TSA. The initial increase in up-regulated

genes was significant, i.e., 8964 genes from whole bone marrow in day 3 cultures after TSA treatment. However, there was not much difference after removal of TSA, from day 3 cultures to day 10 cultures in the presence of high glucose and GLP-1. One explanation is that other factors might now be controlling the expression of the genes: the differentiation of the BM cells into islet-like cells is complete, and now only the housekeeping genes and the specific genes involved with insulin production are functional. However, further work is required to clear up this point.

5.4 Expression of pancreas-specific hormones in differentiated cells

Endocrine pancreas contains alpha cells, beta cells, delta cells and PP-secreting cells, which secrete the major hormones glucagon, insulin, and somatostatin and PP, respectively. Insulin and glucagon are major hormones that regulate the level of glucose in the blood. Whether the differentiated BMSC contained these hormone-producing cells was investigated by means of immunocytochemistry.

The most essential feature of the *in vitro* differentiated BMSC is insulin production. To prove that insulin present in differentiated BMSC stems from intracellular synthesis co-expression of insulin and C-Peptide was analyzed. It is necessary to confirm whether the cells synthesize the insulin or only absorbed insulin from the medium. C-Peptide is a by-product of insulin synthesis, and therefore the demonstration of C-Peptide and the co-localization of insulin are reliable methods for investigating pancreatic differentiation *in vitro*. The formation of C-Peptide demonstrates *de novo* insulin production and excludes those cells that only concentrate insulin from the medium. A high fraction of insulin-positive cells in islet-like cells co-expressed C-Peptide and were organized in islet-like

clusters. Co-expression of insulin and C-Peptide in most of the BMSC-derived cells indicated *de novo* insulin synthesis and ruled out the idea that the cells took insulin up from the culture medium.

In addition to insulin-producing cells, some somatostatin-positive and glucagon-positive cells were present in culture. Analysis by single-staining immunocytochemistry showed that in contrast to the large number of insulin-positive cells fewer glucagon and somatostatin positive cells were also present in culture. Double-immunostaining for insulin and somatostatin confirmed the presence of a minor fraction of somatostatin-containing cells. These results are in agreement with endocrine pancreatic tissue, viz., 60-80% beta cells (insulin secreting) and only 3-10% delta cells (somatostatin secreting). The presence of cells positive for insulin, glucagon, and somatostatin cells confirmed the presence of endocrine pancreas specific beta, alpha and delta like cells in our culture system, similar to cells in pancreas various cell types result from *in vitro* differentiation conditions.

The expression of transcription factor PDX 1, which is necessary for early pancreatic development, was also analyzed in the islet-like cells. Although, at later stages, high levels of the PDX 1 gene transcript are found, we have failed to detect PDX 1 by immunocytochemistry. This is not surprising because, as shown previously, even when the mRNA for PDX 1 is highly abundant, immunoreactivity can hardly be detected (Stoffers et al., 1999).

During embryogenesis, only those cells expressing transcription factor Ngn3 are islet progenitors (Gu et al., 2002). Indeed, we have revealed the expression of the Ngn3 exclusively in the nucleus of our islet-like cells. Other reports (Chiang et al., 2003) have

shown that precursor cells co-expressing glucagon, insulin, and Ngn3 eventually become mature β -cells. The islet-like clusters in our culture system express insulin, glucagon, and Ngn3 and thus resemble pancreatic precursor cells with respect to these parameters.

We have compared the immunohistochemical staining of adult mouse pancreas with that of our islet-like cells. Insulin and C-peptide staining in murine adult pancreas was similar to that in the clusters of islet-like cells, whereas the somatostatin- and glucagon-positive cells formed the mantle of the islets of Langerhans. The 16.5-day embryo was used as a positive control for transcription factor NGN-3 as it is not expressed in adult pancreas tissue. As a whole, the immunohistochemical staining pattern of BMSC-derived islet-like cells was similar in nature to that of the islets of Langerhans and endocrine specific cells were present in the differentiated BMSC.

Co-expression of insulin and C-Peptide showed the *de novo* synthesis of insulin by islet-like clusters. Three-dimensional (3D) reconstruction images were created for islet-like clusters stained with anti-insulin antibody and anti-C-Peptide antibody, by using confocal microscopy. The images showed the perfect co-localization of insulin and C-Peptide. Further, insulin and C-Peptide staining was granular, suggesting a location in secretory granules. Most of the insulin-containing granules were situated near the periphery in the cytoplasm, without any background in the nucleus. 3D reconstruction images thus confirmed the co-localization of insulin and C-Peptide in the differentiated islet-like cells. These cells were full of insulin-containing granules situated near the cell periphery and so could be considered as mature and able to secrete insulin upon high glucose induction.

5.5 Regulation of insulin secretion in differentiated islet-like cells

A fundamental property of pancreatic β -cells is to regulate the secretion of insulin in response to glucose levels. BMSC cultured in presence of TSA for 3 days and subsequently cultured in a high glucose medium with 10 nm GLP-1 formed islet-like clusters. Glucose-regulated insulin release was analyzed, by means of ELISA, in the differentiated islet-like cells *in vitro* by exposing the islet-like cells to high glucose. The islet-like cells, after stimulation with high glucose, secreted significant amounts of insulin. The different concentrations of glucose therefore induced insulin release in a glucose-dependent manner. Although this secretion of insulin seems to be dependent upon the concentration of glucose in the medium, osmotic effects cannot be ruled out, as the insulin secretion was measured at supra physiological conditions. However, Lumelsky et al., (2001) have previously found no such effects with sucrose. In contrast, control BMSC cultured in normal medium showed no significant release of insulin in the presence or absence of glucose challenge. These data suggest that high glucose culture plays an indispensable role in the differentiation of BMSC into insulin-producing cells, and that differentiated BMSC are responsive to glucose challenge. GLP-1 is also known to stimulate insulin secretion. Therefore culture conditions; high glucose and GLP-1 are necessary for differentiation of BMSC into functional insulin-secreting cells. In the present study, we have thus successfully demonstrated the secretion of insulin from BMSC-derived islet-like cells by using ELISA.

The differentiated BMSC or islet-like cells synthesize and store detectable amounts of insulin, as determined by immunoprecipitation and Western blot analysis. We have found that the cell lysates of cells cultured for 3 days in the presence of TSA and differentiated

islet-like clusters cultured for 10 days (3 days in the presence of TSA + 7 days in high glucose and GLP-1) contained significant amount of insulin. This correlates with the gene expression data, the insulin gene being up-regulated after 3 days of TSA treatment and in differentiated islet-like cells cultured for 10 days. However, BMSC cultured in normal medium alone show no insulin reactivity. These results suggest that the BMSC-derived islet-like cells store insulin and regulate secretion of insulin in response to glucose challenge.

5.6 Presence of insulin in secretory vesicles of differentiated BMSC

Pancreatic β -cells synthesize and store insulin in secretory vesicles; upon high glucose induction, this insulin is secreted into the blood. Ultrastructural analysis of BMSC-derived insulin-producing clusters was performed to locate the insulin inside the cells. Electron microscopy of differentiated BMSC at low magnification revealed structures typical of secretory cells, with secretory vesicles containing dense granules. Immunogold electron microscopy further showed insulin within the small secretory vesicles of the insulin-producing clusters. Gold-labeling also detected faint globular structures of differing size filled with a low-density material, and granules with an electron-dense core at the apical pole of the islet-like cells. In comparison, adult pancreatic β -cells also exhibited several positive immunogold particles per secretory granule. Control BMSC cultured in normal DMEM possessed a few non-specific particles in the cytoplasm but no specific labeling. Therefore differentiated BMSC thus exhibited features typical of an adult β -cell, and insulin granules were observed within the secretory vesicles suggesting

that these cells are capable of synthesizing and storing insulin similar to pancreatic β -cells.

5.7 Effect of TSA treatment on cells

The higher order chromatin structure represents an important and general regulatory mechanism for gene expression. It can be classified into two cytologically distinct confirmations: relatively uncondensed euchromatin and much denser heterochromatin. Heterochromatin is transcriptionally less active than euchromatin. TSA induces a more “open” chromatin state in response to histone acetylation and increases the accessibility of transcription complexes to genomic DNA (Gorisch et al., 2005).

The chromatin accessibility changes following TSA treatment were visualized. Nuclei of untreated BM cells were seen to contain dense chromatin areas as brighter spots around the nucleolus and close to the nuclear membrane. However, few cells showed a homogeneous morphology, suggesting that these cells could be stem cells present in the BM (day “0” Figure 4.8). The chromatin distribution of TSA-treated cells was more homogeneous. The chromatin decondensation attributable to acetylation was however reversible. These effects were well demonstrated in cultures of BM cells treated with TSA for 3 days, which contained only a few cells with dense chromatin. After removal of TSA from the culture medium, all cells regained dense chromatin.

5.8 Out look

We have explored the possibility of using mouse BM cells as a source for insulin-producing cells following treatment with TSA and subsequent culturing in the presence of high glucose and GLP-1. GLP-1 is capable of converting intestinal epithelial cells into functional insulin-producing cells. We have generated functional insulin-producing cells from BM cells and have confirmed the occurrence of insulin production by RT-PCR, immunofluorescence, Western blot, and electron microscopy combined with immunogold anti-insulin labeling. Furthermore, the functionality of the insulin-producing cells generated *in vitro* from BMSC has been tested by measuring insulin release in response to high glucose concentrations. Taken collectively, these studies provide evidence that the BM contains pluripotent cells capable of being reprogrammed *in vitro* by TSA to become functional insulin-producing cells.

Here, we showed that bone-marrow-derived can generate pancreatic β -like cells, and that they secrete insulin in a glucose-dependent manner. Diabetes is a leading cause of morbidity and mortality in industrialized countries today. The use of bone marrow as a source of pancreatic cell progenitors has the potential for *ex vivo* expansion, differentiation, and autologous transplantation. Thus, immunosuppression to prevent rejection could be avoided, and further, these cells should not become malignant. The identification of the subpopulation in the bone marrow that gives rise to functional insulin-secreting cells, the mechanism of islet engraftment, and the environmental signals that trigger differentiation will be essential for exploiting these cells for the treatment of type 1 and possibly some forms of type 2 diabetes. However, further research has to be

carried out on chromatin remodeling factors in order to be able to understand the mechanisms that are involved in cell-fate determination. The islet-like cells that have been produced here might provide a suitable model for such research.

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